7. Further studies on the Kik phenotype were aimed at identifying plasmid DNA regions determining this phenotype. For this purpose, the N group plasmid called pCU1 was used as a restriction map of the plasmid was available and some of the plasmid functions had been correlated with the physical map. Transposon Tn5 mutagenesis and mapping of the inserts led to the following conclusions. Tn5 insertions in the *tra* region (abolishing conjugative transfer to *E. coli*) also inactivated the Kik phenotype consistent with a role for conjugative transfer in the Kik phenotype (Thatte *et al.*, 1985b). However, Tra proteins are unlikely to be directly involved with the Kik⁺ phenotype because of the following observations. When insertion derivatives in any of the genetic complementation groups of the *tra* region (Fig. 1) were artificially mobilized into *Klebsiella*, each of them without exception displayed the Kik⁺ phenotype (Rotheim *et al.*, 1988). This was consistent with a hypothesis that Kik⁺ was an intracellular phenomenon in *Klebsiella*, the role of conjugative transfer being to provide an efficient means of delivery of the plasmid into *Klebsiella*.

8. The studies of Thatte *et al* (1985b) implicated two regions on pCU1 which they called *kikA* and *kikB* (both outside *tra*) in the Kik phenotype (Fig 1). The *kikA* region was identified by the restriction mapping of three Tn5 insertions and of deletions derived from the insertions all of which rendered the donor cell Kik⁻ although they were Tra⁺. Kik⁻ Tn5 insertions were not found in the *kikB* region despite intensive transposon mutagenesis of this region. Nevertheless, a comparison

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plasmid pCU1 needed for the establishment in *Klebsiella oxytoca*
of immunity to killing by the same plasmid

by

Margarita Rodríguez de González, M. Sc.

A thesis submitted to the
Faculty of Graduate Studies and Research in partial
fulfilment for the degree of
Doctor of Philosophy

Department of Biology

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of immunity to killing by the same plasmid**

submitted by

Margarita Rodríguez de González

In partial fulfilment of the requirements for the
degree of Doctor of Philosophy

Carleton University

Chairman, Department of Biology
Supervisor
External Examiner

Abstract

When *E.coli* carrying the conjugative plasmid pCU1 is introduced by conjugation into *Klebsiella oxytoca*, 90-99% of the recipients lose their viability and 1-10% survives while in similar reaction with *E. coli* as the recipient no loss of viability is observed. This host-specific killing phenotype is called Kik (killing of *Klebsiella*). The survivors are immune even when the plasmid is cured and no part of the plasmid DNA can be detected in them. It has been inferred that the plasmid acts as a mutator. A region of 1.39 kb adjacent to the known transfer region of this 39 kb plasmid is sufficient to give rise to the immune *Klebsiella* and a number of derivatives carrying this region give rise to immune and non-immune *Klebsiella* at a nearly constant ratio of 8.8 to 1.2. Plasmid DNA from the immune *Klebsiella* when re-transferred to non-immune *Klebsiella* mutate them to the immune phenotype at the same frequency. Plasmid DNA from the non-immune *Klebsiella* are found to have undergone large deletions, insertions by IS1 and IS10-like sequences or point mutations in a new gene called *kikC*. In *Klebsiella* that have been made immune or in *E. coli* that is naturally immune, the lethal effect of *kikC* is overcome by a gene that maps close by and called *korC*. In non-immune *Klebsiella*, *korC* is insufficient for protection against the lethality of *kikC* and it must be also mutated to the immune state. The relationship between the complex structure and function of the *kikC-imm-korC* region is discussed and speculated upon.

Two other regions on pCU1 called *kikA* and *kikB* have been previously suspected to have a joint role in the Kik' and immune phenotype. It is shown that *kikB* has no direct role and that in the absence of this region, the plasmid is structurally unstable in *Klebsiella*. The *kikA* region has a role in the Kik' phenotype but not in the mutator phenotype.

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List of Abbreviations

-	Absence of phenotype
A	Adenine
Ap	Ampicillin
ATP	Adenosine triphosphate
<i>bom</i>	basis of mobility
bp	base pair (s)
BSA	Bovine serum albumin
C	Cytosine
°C	temperature in degrees Celsius
Ci	Curies(s)
Cm	chloramphenicol
CsCl	Cesium chloride
dGTP	Deoxyguanosine triphosphate
dITP	Deoxy-inosine-5'-triphosphate
dNTP	Dideoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-(β -amino-ethylether)N,N'-tetraacetic acid

F ⁺	F plasmid-bearing
F ⁻	Devoid of an F plasmid
F'	F plasmid carrying chromosomal DNA
G	Guanine
g	acceleration due to gravity
gm	gram(s)
h	hours(s)
Hfr	High frequency of recombination
Imm ⁺	Immunity to killing
Imm ⁻	Sensitivity to killing
Inc	incompatibility
IS	Insertion sequence
kb	kilobase
kDa	kilodalton
<i>kik</i>	gene(s) for killing of <i>Klebsiella oxytoca</i>
Kik	phenotype of <i>kik</i> genes
<i>kil</i>	gene(s) lethal to <i>Escherichia coli</i>
Km	Kanamycin
<i>kor</i>	gene(s) required to override the effect of <i>kil</i> gene(s)
λ	Bacteriophage Lambda
LB	modification of Luria broth

M	molar (moles per litre)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar (10^{-3} moles per litre)
mRNA	messenger RNA
MW	Molecular Weight
ng	nanogram(s)
nm	nanometre(s)
OD	optical density
ORF	open reading frame
+	positive phenotype
+/-	presence or absence of phenotype
polA	E. coli DNA polymerase I gene
<i>oriT</i>	origin of transfer replication
PEG	polyethylene glycol
r	resistance
R ⁺	R plasmid-bearing
R ⁻	Devoid of R plasmid
Rep	Replication region
Rif	rifampicin

RNA	ribonucleic acid
sec	second(s)
SDS	sodium dodecyl sulphate
Sp	spectinomycin
Sm	streptomycin
T	Thymine
TAE	Tris-acetate, EDTA buffer
TBE	Tris-borate, EDTA buffer
TE	Tris-HCl, EDTA buffer
TES	Tris-HCl, EDTA, sodium chloride buffer
Tc	tetracycline
Tn	transposon
<i>tra</i>	conjugative transfer gene(s)
Tra ⁺	Transfer proficient
Tra ⁻	Transfer deficient
μCi	microcurie(s)
μg	microgram(s)
μl	microlitre(s)
UV	ultraviolet
V	volt(s)
v/v	volume per volume

w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
%	percent
<	less than
>	greater than

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Introduction

The genetic information in bacterial cells is often located in a single double-stranded DNA molecule, its chromosome. Additionally, many bacteria contain extrachromosomal elements called plasmids that exist in harmony with the bacterial host. Plasmids have been found in most groups of the eubacteria (for review see Bukhari *et al.*, 1977); those that have been found in *Escherichia coli* or found in other eubacteria and then found to be transmissible to *E. coli* are now receiving relatively more attention. The use and misuse of antibiotics during and after the second world war has also contributed to the prevalence of plasmids in *E. coli* and its close relatives. Many of these plasmids carry one or more genes specifying resistance to antibiotics and have therefore been called R (resistance) factors or R plasmids. Most plasmids that have been found in the eubacteria consist of covalently closed circular DNA molecules although recent observations have also uncovered linear DNA plasmids (Sakaguchi, 1990; Hinnebusch and Barbour, 1992).

Traditionally, some plasmid genes have been considered to be essential for the plasmid and others to be non-essential or optional. Thus genes that promote the replication and survival of the plasmid have been considered to be essential, while genes that coded for resistance to antibiotics, heavy metals, the production of toxic substances like bacteriocins, virulence factors, or that promote plant gall formation, nitrogen fixation and degradation of organic compounds have been consider non-essential or optional (Eberhard, 1989). However, it must be noted that such a categorization is not absolute. Plasmid genes placed in the category of 'essential' are

usually so-placed on the basis of experiments in *E. coli*. It is conceivable that some plasmid genes that are not essential for stable maintenance in *E. coli* K12 are essential for maintenance in another bacterium. The converse may also hold. Genes placed in the second category would of course be essential to the host under a particular circumstance e.g. antibiotic resistance genes in an antibiotic-rich environment. Plasmid genes that promote its conjugative intercellular transfer or mobilization may not be essential to the survival of the plasmid (or its host) under laboratory conditions but they may have a significant role for its survival in nature.

Since a plasmid is an intracellular genetic element, the distinction between the need of a plasmid gene for its own maintenance and its need for the maintenance of the host cannot sometimes be unambiguous. This can be illustrated by describing a set of genes found to be associated with several eubacterial plasmids and analogues of which may be of common occurrence. It has been found that plasmid DNA containing certain genes called *kil* cannot be cloned in *E. coli* except in the presence (*in cis* or *in trans*) of other regions of the same plasmid called *kor* (kill over ride). Reciprocally, and in the parental plasmid, *kor* cannot be inactivated without also inactivating *kil*. Our understanding of the mechanism of action of these genes or gene pairs is poor but available evidence suggests that it is the viability of the host that is affected (Figurski *et al.*, 1982; Winans and Walker, 1985a; Kendall and Cohen, 1987). Further examples will be presented and described in a comparative way in the "Literature Review" and Discussion chapter of this thesis.

Their existence raises the possibility that similar gene pairs exist but are operative in one species but not in another, into both of which the plasmid may be transferred.

Two grouping schemes have been used to classify the R plasmids of *E. coli* and its close relatives. One of these schemes uses the components of plasmid DNA that constitute its replicon as defined in *E. coli*. When two plasmids or plasmid replicons co-exist stably in a cell, they are said to be intracellularly compatible implying that each makes available in the cell its own replicon-specificity-determinants. By contrast, when two plasmids or plasmid replicons are incompatible, it is implicit that they compete for a common and limiting plasmid factor for plasmid maintenance. About thirty plasmid incompatibility groups of plasmids have been recognized in *E. coli* (Couturier *et al.*, 1988). A second way in which the R plasmids have been grouped is on the basis of the intercellular conjugative transfer system (abbreviate *tra*) they specify. Conjugation and conjugative transfer are complex processes involving several genes. Most *tra* systems of *E. coli* include a set of genes for the synthesis, assembly and function of cell-surface organelles called conjugative pili which are essential for conjugation. These pili (e.g. N group pili) also provide sites for the attachment of particular bacteriophages (e.g. IKe, PRD1) so that phage-specificity can be diagnostic of a particular conjugative system. The natural plasmids that are the subject of this study are all conjugative and specify both an N group incompatibility system and an N group *tra* system. However, it will

become evident that the association between N *tra* and N incompatibility is not obligatory. A *tra* system of one plasmid (e.g. N *tra*) can be artificially linked to a heterologous replicon and be fully functional. The N pili have been observed to be short, stubby and attached to cell surfaces in few numbers (Bradley, 1979). This may be a reason why interbacterial matings mediated by the N *tra* system are highly efficient only on solid surfaces and not in liquid media, a property shared by at least two other *tra* systems called P and W, each of which also specify short and stubby pili. The N, P and W pili are however distinguishable from one another serologically or on the basis of sensitivity to pilus-specific bacteriophages (Khatoon *et al.*, 1972; Stanisich, 1974; Morris *et al.*, 1980).

Previously, it has been found that all available naturally occurring plasmids of the N incompatibility and *tra* groups display a phenotype called Kik (Killing of *Klebsiella*). This phenomenon is also a property of naturally-occurring W and P group plasmids (Rodríguez and Iyer, 1981), but there is no implication that the phenotype is manifested by a common mechanism by all three groups. The observation was made that when conjugative donors (usually *E. coli* donors) carrying a plasmid of the N group was mated with *Klebsiella oxytoca* (previously called *Klebsiella pneumoniae* see Yocum, 1989) on a solid surface of a nutrient medium for a limited period and then assayed for surviving and viable *K. oxytoca*, 90 to 99% of the recipient *Klebsiella* did not survive and form colonies on a medium selective for all the *Klebsiella*.

The following additional observations on this phenotype and particularly the basis of survival of the minority of the *Klebsiella* motivated the present studies.

1. The Kik⁺ phenotype was not due to a product that diffused from donors cells and to which the recipients were susceptible as could be the case for many antibiotics or bacteriocins (Rodríguez and Iyer, 1981)
2. With naturally occurring plasmid-carrying donors, it required physical contact between the donors and recipients (Rodríguez and Iyer, 1981)
3. The phenotype was inhibited by nalidixic acid, an agent known to inhibit stable plasmid DNA transfer from the conjugative donor to the recipient (Gill and Iyer, 1982).
4. The phenotype was not observed following matings between *E. coli* strains. It is a host specific phenotype (Rodríguez and Iyer, 1981).
5. Progeny of the plasmid-carrying *Klebsiella* selected as transconjugants (and which therefore survived Kik action) could display the Kik⁺ phenotype when used as donors in a second mating reaction with a distinguishable *Klebsiella* as the new recipient (Rodríguez and Iyer, 1981).
6. When the above *Klebsiella* strains that survived were apparently cured of the Kik⁺ plasmid (i.e. no longer carried plasmid markers) they continue to display resistance to Kik⁺ action although they were good conjugal recipients (Gill, 1985; Rotheim, pers. commun; Rodríguez, this study). This resistance is therefore best described as an immunity reaction with an intracellular basis.

7. Further studies on the Kik phenotype were aimed at identifying plasmid DNA regions determining this phenotype. For this purpose, the N group plasmid called pCU1 was used as a restriction map of the plasmid was available and some of the plasmid functions had been correlated with the physical map. Transposon Tn5 mutagenesis and mapping of the inserts led to the following conclusions. Tn5 insertions in the *tra* region (abolishing conjugative transfer to *E. coli*) also inactivated the Kik phenotype consistent with a role for conjugative transfer in the Kik phenotype (Thatte *et al.*, 1985b). However, Tra proteins are unlikely to be directly involved with the Kik⁺ phenotype because of the following observations. When insertion derivatives in any of the genetic complementation groups of the *tra* region (Fig. 1) were artificially mobilized into *Klebsiella*, each of them without exception displayed the Kik⁺ phenotype (Rotheim *et al.*, 1988). This was consistent with a hypothesis that Kik⁺ was an intracellular phenomenon in *Klebsiella*, the role of conjugative transfer being to provide an efficient means of delivery of the plasmid into *Klebsiella*.

8. The studies of Thatte *et al* (1985b) implicated two regions on pCU1 which they called *kikA* and *kikB* (both outside *tra*) in the Kik phenotype (Fig 1). The *kikA* region was identified by the restriction mapping of three Tn5 insertions and of deletions derived from the insertions all of which rendered the donor cell Kik⁻ although they were Tra⁺. Kik⁻ Tn5 insertions were not found in the *kikB* region despite intensive transposon mutagenesis of this region. Nevertheless, a comparison

Figure 1. Diagram illustrating the structure of the plasmid pCU1 with emphasis on the regions relevant to this thesis. *Tra* is the region needed for conjugative transfer of the plasmid in matings between *E. coli*. It is delineated into its known genetic complementation groups as established by the analysis of Thatte *et al* (1985a). Of these known complementation groups, only A to F are needed for the synthesis of the N pilus. The dotted box represents the *oriT* region (Paterson and Iyer, 1992). The location of the *kikA* region has been shown previously (Thatte., *et al* 1985b; Hengen *et al.*, 1992a) and the region is now well-characterized (Holčík, pers. commun). *kikB* has been defined only by the difference in behaviour of the two pACYC184 clones, pCU109 (with coordinates 27.7 to 10.2) and pCU101 (with coordinates 27.7 to 8.7). Other symbols are self explanatory (see also the list of abbreviations).

of two clones in the plasmid vector pACYC184 (Fig 1, pCU109 and pCU101) showed that pCU109 was Kik⁺ while pCU101 was either Kik⁻ in some experiments or weakly Kik⁺ in others (Thatte, 1984; Rotheim, pers. commun). They therefore suggested that both *kikA* and *kikB* regions are necessary for the full expression of this phenotype.

9. Hengen *et al* (1992a) cloned and sequenced the *kikA* region of 500 bp (Figure 1) and placed this region downstream of the inducible *tac* promoter in the cloning vector pJF118HE. The resulting plasmid called pPH4 has been found to transform *Klebsiella* as efficiently as its vector. *Klebsiella* carrying pPH4 has the same growth rate as *Klebsiella* carrying the related vector (Holčík, pers. commun). Hengen *et al* (1992a) and Holčík (pers. commun) have also found pPH4 to be maintained stably in *Klebsiella*. However, when this *Klebsiella* carrying pPH4 was exposed to a high concentration of IPTG, viability was lost (Hengen *et al.*, 1992a). Thus, at least in this artificial system, conjugation could be entirely bypassed and *kikA* alone was sufficient to kill *Klebsiella*. The possible mechanism of action of *kikA* is currently being studied by M. Holčík in this laboratory. When *kikA* in pPH4 is induced by IPTG, survivors that are immune to the Kik⁺ phenotype have not been observed to arise. This is in contrast to the observation that when a plasmid clone like pCU109 (Figure 1) is introduced into *Klebsiella*, the *Klebsiella* carrying pCU109 is immune to the Kik⁺ phenotype and continues to be immune even when the plasmid is

apparently cured from the strain. Thus, some other plasmid region present in pCU109 and absent in pPH4 is necessary for the emergence of immune *Klebsiella*.

The major objective of this study was to locate this region(s) on the plasmid and to characterize it in order to understand how *Klebsiella* displaying this form of immunity emerge from an initially sensitive population. A second objective was to address the question of how the *kikB* region was contributing to both the KiK' phenotype (Thatte *et al.*, 1985b) and to the immunity phenotype as the experiments of Gill (1985) had indicated. Experiments that bear on both these questions are presented as Part I and Part II of Results. In the chapter that follows this Introduction, other examples of both plasmid-determined killing of bacterial hosts and of immunity to this killing are reviewed. The last chapter is an interpretative discussion of the results of experiments in this thesis as well as a comparison to the observations presented in the review of the published literature.

Literature Review

Several plasmid-mediated killing mechanisms and their corresponding immunity systems have been described. Most of them were studied in *Escherichia coli*. The killing of plasmid-free cells is a widely used strategy in the plasmid world. There is both killing by the release of a toxic substance into the medium and also killing from within without a toxic substance being released extracellularly. This review will be concerned with known mechanisms of killing and how immunity to the different kinds of killing is achieved.

BACTERIOCINS

Many plasmids carry genes for the production of antibiotic substances called bacteriocins; they are produced by different bacterial genera among the Gram negative and Gram positive eubacteria (for a review see Konisky, 1982). Bacteriocins are distinguished from antibiotics by their narrow range of host-specificity; they are active only on strains related to the producing strain or species. The best-studied bacteriocins are the ones produced by enterobacterial strains which have been classified into two groups: Colicins (large polypeptides from 30 to 80 kDa) and Microcins (small polypeptides of < 10 kDa). The mode of action of these toxic molecules on sensitive strains involves a sequence of molecular interactions, initiated by the adsorption of the colicin molecule to specific outer membrane protein receptors, translocation across the membrane and terminating by the direct interaction of the bacteriocin with its particular target inside the cell

(Konisky, 1982). The receptors are utilized by the colicin molecule in order to overcome the physical barrier imposed by the membrane and to gain access to the target. Some of these receptors have been identified as specific membrane proteins that participate in the transport of external substrates. Di Masi *et al* (1973) have shown that the receptor for the E1, E2 and E3 colicins is the same as the receptor that is utilized for vitamin B₁₂ uptake. The colicin K receptor serves as a specific diffusion pathway for nucleotides (Hantke, 1976) and the receptors for colicin M, I and Ib are involved in iron metabolism (Wayne *et al.*, 1976; Konisky *et al.*, 1976). Even though the presence of the receptor renders the bacteria sensitive to colicin action, their association with other important functions seems to be the selective pressure involved in their maintenance.

With regard to the interaction of the colicin with the target, colicin action can be subdivided into several classes. Some colicins kill sensitive bacteria by forming voltage-dependent ion channels, thus impairing the cell's energy potential. Examples of this type are colicins A, E, K, Ia, Ib, B and N (Davidson *et al.*, 1984). A second class has endonuclease activity; Colicin E2 causes both single and double-strand breaks of the DNA in sensitive cells and colicins E3 and DF13 (formally called cloacin) cuts the 16S RNA of the 30S ribosomal subunit at a specific site near the 3'- terminus (de Graaf *et al.*, 1973). There is a third class with a unique mode of action; colicin M causes lysis of sensitive cells and induces formation of spheroplast by inhibiting peptidoglycan biosynthesis (Schaller *et al.*, 1982).

Colicinogenic strains are immune (insensitive) to the antibiotic they produce; beside the colicin gene each naturally occurring Col plasmid carries two more genes, the immunity and lysis gene. The immune product protects the producing strain against the colicin it produces and the lysis gene is apparently responsible for the translocation of the colicin from the bacterial cytoplasm to the extracellular environment (Pugsley and Schwartz, 1984). These genes are located in the same operon with one of two types of organization: Type A; the colicin, the immunity and lysis genes are located adjacent to each other being co-ordinately transcribed from one common inducible promoter containing two units of the SOS box with the LexA binding site, followed by the three structural genes. Type B; the immunity gene although adjacent to the colicin gene is functionally independent; transcription leading to immune mRNA occurs in the opposite direction to that for the colicin (Pugsley and Oudega, 1987).

The best-studied colicins are those with nuclease activity. Studies on the function of these colicins in relation to their structure has revealed that they possess various domains with specific functions: an amino-terminal domain involved in translocation of the colicin molecule across the envelope of susceptible cells, a central domain with receptor binding activity and a carboxyl terminal domain possessing *in vitro* bactericidal activity. The immunity proteins are specific inhibitors of the respective colicin nucleases and protect the colicinogenic host cell from the killing action of both the extracellular and intracellular colicin molecule. Immunity is

defined by a specific interaction between the colicin and immunity proteins. In colicin E2, E3, and E8 the C-terminal fragment obtained by tryptic digestion (referred to as T2A) has a dual function, enzymatic activity and binding to the immune protein. The T2A domain binds specifically to the immune protein and both are released from the cells as equimolar complexes (Toba *et al.*, 1988).

In colicins whose mechanism of killing is the formation of an ion-permeable pore in the cytoplasmic membrane (such is the case of colicin A and E1), they are released in free form into the medium. The related immunity protein has been located in the cytoplasmic membrane (Geli *et al.*, 1989) but at present it is not known how immunity is being accomplished by these colicins.

The protection of colicin M producing cells by the immunity protein was studied by Ölschläger *et al* (1991); colicin M as a pore-forming colicin shares the cytoplasmic membrane as the target site and this is also the location of the immune protein. Three working hypotheses were presented by the authors in order to explain immunity to colicin M.

1. immunity may be due to the binding of the colicin to its immune protein leading to an enzymatic modification of the colicin.
2. Immunity protein could interact with the target inhibiting the lethal effect of colicin M.
3. The immune protein could interfere with the receptor or the translocation of the colicin from the receptor to the target. The results indicated that the most likely

mode of inhibition is that of a direct interaction with the colicin and the immune protein. The authors demonstrated that a surplus of colicin over immune protein (the immune gene was cloned in a one-copy plasmid) by enhancing the colicin transport to sensitive cells render the cells sensitive. Strains containing two copies of the immunity region were still sensitive to colicin M, but the introduction of the immunity region on a multicopy plasmid (20 copies) reestablished immunity. The data presented by the authors are consistent with the hypothesis of inactivation of the colicin by the immune protein (anchored in the cytoplasmic membrane) before the colicin reaches its target.

MICROCINS, LOW MOLECULAR WEIGHT BACTERIOCINS

Microcins are dialysable low molecular weight antibiotics (generally < 10 k Da) produced and excreted by enterobacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. Unlike most colicins, the production of microcins is not stimulated by DNA-damaging, SOS-inducing agents and they are synthesized during stationary phase. As with colicins, microcins and their immune protein are encoded by plasmids with the exception of microcin MccH47, where the microcin producing and immunity genes are located in the chromosome (Laviña *et al.*, 1990). Microcin-producing strains were classified by cross-immunity, biochemical and genetic criteria in five groups: A, B, C, D and E. Type A inhibits metabolic enzymes, type B blocks DNA replication, type C inactivates protein synthesis, type D impairs the cell's

energy generating system and type E disrupts the transmembrane electrical potential (Baquero and Moreno., 1984).

The best-characterized microcins are C7 and B17. Microcin C7 (MccC7) is produced by a 43 kb plasmid (pMccC7) of the IncX incompatibility group. It is a linear heptapeptide (1,000 Da) with modifications at the N and C terminal end of the molecule; it is a bacteriostatic agent that blocks protein synthesis (García-Bustos *et al.*, 1985). The genes responsible for the production of the microcin and immunity to it have been localized by transposon mutagenesis to a DNA segment of approximately 5 kb. This DNA has four regions defined as α , β , γ and δ which are transcribed in the same direction. The γ region may encode the structural gene for microcin C7, but α is also required for microcin production. The regions β and γ confers immunity to the producing strain (Novoa *et al.*, 1986). There is a second mechanisms of immunity provided by a chromosomal gene located at 57.5 min on the *E. coli* map and designated *mprA* (for *microcin production regulation*). The MprA product is a 19 kDa polypeptide which inhibits the production of microcin C17 and other small-peptide antibiotics like ColV and MccB17 (del Castillo *et al.*, 1990). Interestingly, production of microcin MccC7 is activated by the product of a gene located at 59.5 min (the *appR* locus) on the *E. coli* map. AppR product controls MccC7 production by activating the transcription of the adjacent α and β regions of the microcin producing region (Díaz-Guerra *et al.*, 1989). This is an

example of a plasmid gene which utilizes a regulatory molecule encoded for the chromosome to control its own transcription.

Another well characterized microcin is microcin B17, a glycine-rich peptide of approximately 3.200 Da, encoded by a 70 kb conjugative plasmid (pMccB17) of the incompatibility group Inc F2. Microcin B17 (MccB17) inhibits DNA replication in sensitive bacteria leading to induction of the SOS repair system, massive DNA degradation and cell death (Davagnino *et al.*, 1986). Recent studies showed that the MccB17 target is the B subunit of DNA gyrase (Vizán *et al.*, 1991). Seven contiguous genes, *mcbABCDEFG* located in a 5.2 kb DNA stretch are responsible for MccB17 production and immunity in the producing bacteria. The MccB17 production region, contains four genes designated *mcbA*, *mcbB*, *mcbC*, and *mcbD*, (San Millán *et al.*, 1985; Genilloud *et al.*, 1989). The product of *mcbA* is a 69 amino acid precursor that is processed by the action of the *mcbB*, *mcbC* and *mcbD* gene products from an inactive form to the 43 amino acid active product (Garrido *et al.*, 1988). The mechanism by which the *mcbBCD* gene products mediate this modification is not known. Immunity is mediated by the genes *mcbE*, *mcbF* and *mcbG* encoded in almost 2 kb of DNA. In cells producing the microcin the loss of the *mcbG* gene product causes a slower growth rate, SOS induction, cell filamentation and plasmid loss. The products of the *mcbE* and *mcbF* genes constitutes an exporter of microcin B17, the complex of McbE and McbF acts as a membrane 'pump' that exports the microcin to the periplasmic space; indirectly, the

action of these two proteins confers a limited degree of immunity by keeping the intracellular concentration of the microcin low (Garrido *et al.*, 1988). Thus, the immunity system for microcin B17 is composed by two complementary mechanisms; the McbE and McbF action and the protection that the product of *mcbG* exerts on the target of microcin B17 action. Like microcin C7, microcin B17 production is inhibited by the chromosomal regulator gene *mprA* (del Castillo *et al.*, 1990). Cloning of *mprA* on a multicopy plasmid could compensate for the lack of *mcbG* gene in immunodeficient cells, by decreasing the production of the microcin and allowing the McbE/McbF complex to export the toxin before it inhibits the growth of the cell. As in the case of microcin C7, transcription of the *MccB17* genes is also stimulated by chromosomally located genes, one of which is the regulatory protein OmpR (Hernández-Chico *et al.*, 1986). This protein acts as a positive regulator of transcription of the structural genes of the major outer membrane porin proteins OmpC and OmpF of *E. coli* (Hall and Silhavy, 1981). The sequences essential for OmpR-dependent activation lie close to the P_{mcb} promoter upstream of the structural gene (*mcbA*).

KILL SYSTEMS ACTING INSIDE THE BACTERIAL CELL

Killing systems that act at the level of segregation of plasmids appear to work on a poison and antidote principle. The plasmid produces a killer substance and also an agent that blocks its activity; the system mediates plasmid stabilization

by killing plasmid-free segregants (Gerdes *et al.*, 1986; reviewed by Nordström and Austin, 1989).

Two mechanisms have been described in the literature that could account for the post-segregational killing of host cells that do not receive the plasmid on cell-division and the protection from killing of those that received the plasmid. In each case, plasmid loss is followed by decay of the antidote, leaving a lethal protein to induce the cell death.

The *hok/sok* system is located at the *parB* locus in the transfer-leading region of plasmid R1 of the incompatibility group FII (Gerdes *et al.*, 1985). The system consists of two genes: *hok* (host killing) a potent cell-killing protein of 52 amino acids and *sok* (suppressor of killing) a trans-acting antisense RNA that represses *hok* gene expression (Gerdes *et al.*, 1988). The toxin acts from within by damaging the cell membrane with the concomitant drop of the membrane potential and leakiness which results in efflux of cellular Mg^{2+} with the appearance of non-viable cells, which are turned into 'ghost' cells, with a translucent centre and the cell material condenses at the poles (Gerdes *et al.*, 1986). Killing of plasmid-free cells is accomplished through differential decay of a very stable Hok mRNA and an unstable Sok antisense RNA; translation of *hok* is activated after decay of Sok-RNA or after addition of the transcriptional inhibitor rifampicin (Gerdes *et al.*, 1988; Gerdes *et al.*, 1990). Blockage of transcription by rifampicin leads to a rapid depletion of the Sok-RNA pool, leaving the stable Hok mRNA free to be

transcribed. The *hok/sok* locus is very compact, two open reading frames *hok* and *mok* (mediation of killing); the 5' end of the *mok* gene overlaps with the 3' end of the *sok* gene and the 3'-end of *mok* gene overlaps with almost the entire *hok* gene. The 5' end of Sok-RNA recognizes the complementary region in Hok-mRNA at the *mok* TIR (translation initiation region) region (Thisted and Gerdes, 1992). The killer system also produces truncated mRNAs that are shortened at the 3' end by 35 to 70 nucleotides. These are the ones producing the toxin *in vivo*. By using *in vitro* synthesized mRNAs Thisted *et al.*, 1994 (referred by Gerdes, 1994) have shown that the full-length RNA is translationally inactive; the analysis of the secondary structure of this mRNA suggest that the 3'-end encodes an anti-Shine- Dalgarno sequence that folds back onto the *mok* TIR region and represses translation of *mok* and *hok*, creating an extra level of control.

A family of *hok*-homologous killer genes has been identified in plasmids of several incompatibility groups (Nielsen *et al.*, 1991). The F plasmid carries two *hok*-homologous loci: *srnB*, located near the RepFIC region (Ohnishi *et al.*, 1977; Akimoto *et al.*, 1986) and *flm* which is almost identical to *hok*, and is located in the transfer leading region (Goloub and Panzer, 1988; Loh *et al.*, 1988). The *pnd* loci (promotion of nucleic acid degradation) similar to *hok* was found in the IncI α plasmid R483 and the IncB plasmid R16 by their induction with rifampicin (Ohnishi and Akimoto, 1980; Akimoto and Ohnishi, 1982). Two homologues of *hok* were also found in the chromosome of *E. coli*; the *relF* gene located in the *relB* operon

which maps at min 34.4 in the *E. coli* chromosome (Bech *et al.*, 1985) and the *gef* gene which maps at 0.4 min (Poulsen *et al.*, 1989). It was also shown that similar sequences were present in the chromosome of *E. coli* B and *E. coli* C and also in other Gram-negative bacteria like *Rhizobium* and *Agrobacterium* species (Poulsen *et al.*, 1989). Clearly, these systems are not rare.

SYSTEMS WHERE THE KILLER AND BLOCKING AGENT ARE PROTEINS

A F⁺ culture maintains its plasmid not only by periodic plasmid replication and equipartitioning but also by destruction of F⁻ segregants. The existence of this mechanism was first demonstrated by Ogura and Hiraga (1983). They found it to be specified by a locus called *ccd* (control of cell death). The *ccd* operon produces three proteins: CcdA, CcdB and RepD and they are located next to *oriV* in the F plasmid. Jaffé *et al* (1985) elucidated the mechanism of *ccd* action by showing that *ccdB* causes the death of plasmid-free segregants by producing a toxic protein CcdB (11,700 Da). The lethal action of CcdB is prevented by the CcdA (8,700 Da) protein. The mechanism underlying post-segregational killing could be differential decay of the activities of CcdA and CcdB, with the half-life of active CcdA protein being shorter than that of active CcdB product. In plasmid-free bacteria the persistence of the toxin leads to cell death. Tam and Kline (1989) have shown that *ccd* transcription is autorepressed and it requires the complex of CcdA and CcdB

products. CcdB protein always copurified with CcdA. The proteins maintained a complex state of 69,000 Da *in vitro*; the authors also speculated that this complex state could exist *in vivo* and may have a dual purpose: to maximize the concentration of autorepression and to neutralize CcdB cytotoxicity.

Bernard and Couturier (1991) have shown that 41 residues of the C-terminal end of the CcdA protein are sufficient to antagonize the killer activity of the CcdB toxin. As a step in analyzing the molecular mechanism that coordinates DNA replication of F plasmid and cell division of the host bacteria; Miki *et al* (1988) attempted to identify the gene(s) which is the target of the *ccdB* product by isolating bacterial mutants tolerant to inhibition. Temperature-sensitive growth-defective mutants were screened for mutants that escaped the CcdB action when the host carries a *ccdA* mutant F plasmid. Phage P1-mediated transduction and complementation analysis located six out of nine mutants in the *groES* gene, which is essential for the morphogenesis of certain bacteriophages and also for growth of the bacterial cell. These observations suggest that the gene *groES* plays a key role in the coupling between replication of the F plasmid and cell division of the host cell. The other mutants were shown to have mutations in the *gyrA* gene. The nucleotide sequence of one of the mutants indicated a base change from G to A at position 641 of the *gyrA* gene, resulting in a change from glycine to glutamic acid at position 214. Miki *et al* (1992) suggested that the *ccdB* gene product modulates DNA gyrase activity by interacting with the A subunit of DNA gyrase. They demonstrated that

heterozygous cells containing the mutant and the wild type *gyrA* allele were able to survive in the presence of the toxin by using the mutant DNA gyrase. Similar results were obtained by Bernard and Couturier (1992), their mutants were located in the gene encoding the A subunit of topoisomerase II. They have shown that in the wild type strain, the CcdB protein promotes plasmid linearization, whereas in the *gyr* mutant strain which resist the action of CcdB protein, no plasmid linearization was observed.

The plasmid R100 a conjugative plasmid of the incompatibility group FII, closely related to R1 has two genes which are responsible for its stable inheritance during cell division; *pemI* (for *plasmid emergency maintenance*) and *pemK* (Tsuchimoto *et al.*, 1988). The *pemK* gene product, a protein of 11.9 kDa is the killing factor and the product of *pemI* gene, a protein of 9.3 kDa is the inhibitor. These two genes are equivalent to the *kis/kik* genes of the ParD region of plasmid R1 (Bravo *et al.*, 1988; Ruiz-Echevarría *et al.*, 1991). Tsuchimoto *et al* (1992) have shown that the mechanism of killing is due to the rapid degradation of the PemI protein as compared to the degradation of the PemK protein. They analyzed protein stability in Lon⁺ and Lon⁻ strains in the presence and absence of the *novo* protein synthesis. Induction of PemI and PemK proteins simultaneously did not cause inhibition of cell growth, the same treatment with repression of de novo synthesis of both proteins caused cell death in the Lon⁺ but not in the Lon⁻ host, these results also agreed with their observation of PemI protein being more stable in Lon⁻ strains as

compared to Lon⁺ strains. The *lon* gene encodes an ATP-dependent protease involved in degradation of abnormal and also unstable proteins (Gottesman, 1989).

Similar results to the one presented above were obtained by Van Melder *et al* (1994) for the CcdA protein. Using several protease mutant strains they were able to show the stability of CcdA protein in Lon⁺ mutants. In the *in vivo* situation the CcdA and CcdB form a complex that regulates its own synthesis (Tam and Kline, 1989) and the half life of CcdA is longer when CcdB is produced. The authors suggested that partial resistance to proteolysis *in vivo* could arise either from the protein-protein interaction protecting the protease recognition site or from the conformation of the complex.

Bacteriophage P1 which lysogenizes *E. coli* as a low copy plasmid also contains two genes that contribute to plasmid stabilization (Lehnherr *et al.*, 1993). The two genes, *doc* (*death on curing*) producing a 126 amino acid product and *phd* (*prevent host death*) producing a 73 amino acid product are part of one operon where the synthesis of Doc appears to be translationally coupled to that of Phd. The genes of the P1 system are unrelated to the ones described previously, loss of plasmid F leads to cell filamentation and SOS induction; whereas P1 loss leads to a reduction in cell size and a decreased capacity for SOS induction. Preliminary results of the authors suggested that a protease other than Lon is implicated in the P1 system.

***kil/kor* GENES OF BROAD HOST-RANGE PLASMIDS RK2 (IncP), pKM101 and pCU1 (IncN)**

Plasmids of incompatibility group P have a wide host-range. RK2, a plasmid indistinguishable from RP4, RP1 and R18 (Burkardt *et al.*, 1979; reviewed by Thomas and Smith, 1987) and one of the best studied plasmids of this group is capable of replication, stable inheritance and transfer between most gram-negative bacteria (reviewed by Thomas and Helinski, 1989; Guiney and Lanka, 1989). The basis of this promiscuity might be both the ability of the plasmid to replicate in different hosts as well as the existence of additional genes for stable maintenance.

A network of coregulated genes known as the *kil/kor* regulon (Figurski *et al.*, 1982) has been shown to be involved in plasmid maintenance and in broad host range. The *kil* genes include a series of host-lethal genes which cannot be cloned unless a regulatory sequence denoted *kor* (*kil* override) is present in the cell. The loss of cell viability was shown by cloning the *kor* determinant into a ColE1-like plasmid which requires *E. coli* DNA polymerase I for plasmid replication and the *kil* determinant in a vector which is pol I independent. The *kil* and *kor* determinants were established together in these separate vectors at 30 °C in a *E. coli polA* mutant temperature-sensitive for DNA polymerase I. Killing was observed at 42°C even in the absence of selection, indicating that the loss of the *kor* plasmid prevented colony formation.

Four *kil* loci: *kilA*, *kilB*, *kilC* and *kilE* (Figurski *et al.*, 1982 ; Kornacki *et al.*, 1993) and the regulatory *korA*, *korB*, *korC*, *korE*, *korFI* and *korFII* have been described for *E. coli*. An interesting feature of this network is that the *kil* genes are controlled by overlapping combinations of *kor* genes, the exception is the *kilB* locus which is only controlled by the product of *korB*. None of the *kil* genes are essential for replication of the plasmid, but the regulation of the *trfA* gene (essential for plasmid replication) by the products of *korA*, *korB*, *korE* and *korF* (Schreiner *et al.*, 1985) implicates the *kor* genes in the mechanism of replication control of the plasmid. *trfA* codes for two polypeptide products that acting at the origin of replication (*oriV*), initiate replication of the plasmid (reviewed by Thomas and Helinski, 1989).

The *kilA* locus contains three genes *klaA*, *klaB* and *klaC* which encode polypeptides of 28, 42 and 32 kDa respectively (Goncharoff *et al.*, 1991) and are regulated by the *korA*, *korB* and *korE* products (Young *et al.*, 1985; 1987). The lethal effect of *klaA* was analyzed by cloning the gene in a λ transducing phage, λ *pklaA*-1 (Saltman *et al.*, 1991). Bacterial host expressing λ repressor (to prevent lytic growth) in absence of the repressors encoded by *korA* and *korB* were infected with the transducing λ *pklaA*-1 phage. Observation of the *kor*^o infected cell revealed that the unregulated expression of *klaA* produced elongated cells with the inhibition of growth. Cell death is produced by arrest of DNA, RNA and protein synthesis.

The authors consider the possibility that the *kilA* locus encodes accessory factors that facilitate interaction with important host factors acting in DNA replication or gene expression.

Young *et al* (1985) have shown by gene fusion and promoter replacement that the *korA* product controls *kilA* by regulation of gene expression from the *kilA* promoter by acting as a repressor that inhibits initiation of transcription. The target of KorA is a palindromic sequence (5'-GTTTAGCTAAAC-3') at the -10 position of the promoters of *kilA*, *korA* and *trfA*.

The *kilB* locus is the only one that has been implicated in conjugation; a transfer-defective mutant of RP4 was found to have an Tn7 insertion in the *kilB* region (Barth *et al.*, 1978). Plasmids deleted in *kilA*, *kilC* and *kilE* have the same frequency of transfer as the wild type plasmid. The mutation in the *kilB* locus also abolished the sensitivity to bacteriophage PRD1, a phage known to attach to the sex pili of IncP group (Olsen and Shipley, 1973) and eliminated the surface exclusion phenotype. Analysis of the *kilB* locus by Thomson *et al* (1993) indicated the presence of at least three genes *kilA*, *kilB* and *kilC*. The predicted amino acid composition of KilA suggested homology to the product of *virB11* gene from *Agrobacterium tumefaciens* Ti plasmid (a gene involved in transfer of T-DNA to plant cells). The KilB product was predicted to be a membrane protein with homology to the product of the *kilC* lethal gene of the *kilA* operon. The third gene in the *kilB* locus is *kilC*; its function is not known yet but the deduced amino acid

sequence shows a perfect match with a leucine zipper motif. This led the authors to suggest a possible role in the formation of protein complexes needed for the assembly of the sex pilus. The Tn7 insertion in RP4 described above was mapped by Thomson *et al* (1993) in the *klbC* gene. The host-lethal phenotype of the *kilB* operon was located by Motallebi-Veshareh *et al* (1992) in the first gene of the operon (*kilB1*) which corresponded to the *klbA* gene described by Thomson *et al* (1993). Results presented by the later group indicated that *klbA* (*kilB1*) is not sufficient for the host lethal phenotype and that some of the *klbB* gene is needed. They based their conclusion on two plasmid deletion derivatives one containing *klbA*, the promoter and portion of the *klbB* gene and expressed a Kil⁻ phenotype while another deletion derivative which contained the *klbA* and approximately 70% of the *klbB* gene was expressing a Kil⁺ phenotype. *klbC* is not lethal to *E. coli*. The *kilB* operon is regulated by the transcriptional repressor KorB, a protein of 39 kDa (Balzer *et al.*, 1992) that interacts with 13-bp palindromic sequences (TTTAGC_C^GGCTAAA) denoted O_B sequences (Smith *et al.*, 1984). The sequences are present in the RP4 genome 12 times, localized in the maintenance and replication regions as well as the transfer regions Tra1 and Tra2 (Lessl *et al.*, 1992). Williams *et al* (1993) have shown the nature of the *korB* regulation by footprinting and restriction protection, they demonstrated that KorB and the RNA polymerase could bind simultaneously to the O_B sequences. Permanganate footprinting using the *korABF* promoter and purified KorB protein showed that KorB acts by preventing isomerization of the

RNA polymerase-promoter complex, preventing the transition from the closed to open complex.

A fourth *kil* locus denoted *kilE* was found to be regulated by *korA* and *korC*; the nucleotide sequence of *kilE* revealed two potential multicistronic operons: *kleA* with two genes, *kleA* and *kleB* and the *kleC* operon with four genes *kleC*, *kleD*, *kleE* and *kleF* (Kornacki *et al.*, 1993). Expression of the *kleA* operon was lethal to *E. coli* in the absence of their *kor* regulators. The expression of the *kleC* operon gave rise to a *Kil⁺* phenotype with slow growing and unhealthy colonies when *kor* was not present in the cell. The promoter region for the two operons contains two palindromic regions upstream of the -35 sequences which are the predicted target for KorA and KorC proteins. The conservation of the *kor* genes among plasmids of the P incompatibility group (Figurski *et al.*, 1982) and their regulatory function in controlling genes that are involved in replication, transfer and maintenance of the plasmid indicates the importance of these genes even though their specific functions are not completely understood.

Plasmid pKM101 from the IncN group encodes two *kil* loci designated *kilA* and *kilB* which could not be cloned unless their corresponding plasmid-specified *kor* sequences were in the same cell in *cis* or *in trans* (Winans and Walker, 1985a). The authors used the same terminology that had been used previously for the RK2 *kil/kor* genes. Mutation in the *kilB* region was found to produce a transfer deficient plasmid. The mutation was mapped to *traE*, one of the 11 complementation groups

of the transfer region in pKM101 (Winans and Walker, 1985b). *kilA* was located outside the transfer region. Presence of the *kilA* region was found to cause lethality even when *korA* was present. It was found that *korB* was also needed for complete survival. These regions have not been studied further.

kil genes were also found in another plasmid from the same group, the plasmid pCU1 which is the subject of this study. Evidence of two regions in pCU1 that could not be cloned in *E. coli* were presented by Hengen (1991). Localization of these regions occurred when attempts were made to subclone a pCU1 *SalI* restriction fragment from the Tn5 insertions in *kikA* (using the derivative pCU184 and pCU66) to the *SalI* site in pCU1 located at coordinate 39.0 (see Figure 2). The fragments were cloned in pUC19 and the recombinants were designated, pUC19-184A and pUC19-66A to correspond to the parental plasmid. All the pUC19-66A derivatives isolated showed the expected insert of 10.6 kb; curiously, all the pUC19-184A derivatives contained an extra *SalI* fragment from the internal *SalI* site of Tn5 in *kikA*, counterclockwise to the *SalI* site located at coordinate 27.9. This was interpreted by Hengen (1991) as the location of a *kor* region (*korA*), indicating that the extra *SalI* fragment cloned in pUC19-184A was needed for survival in *E. coli*. The *kilA* region was then located from *kikA* to the *PstI* site at coordinate 28.8. A question mark adjacent to *korA* identifies a *PstI* fragment that can be deleted without creating instability. This could place *korA* between coordinates 27.9 (*SalI* site) and 28.8 (*PstI* site).

Figure 2. Mapping of two *kil/kor* regions on pCU1. Preliminary evidence for the existence of regions near *kikA* of pCU1 prohibiting the separation of *kil* and *kor* regions. Transposon Tn5 or Tn5*lac* insertions are shown by circle and stem structures. pCU184, pCU66, and pCU171 are Tn5 insertions within pCU1 designated as 184, 66, and 171 respectively. pNH142 is a Tn5*lac* insertion within pCU109, designated as 142. Arrows indicate the orientation of Tn5 insertion (the direction of the arrow indicates the orientation of the kanamycin resistance promoter). Grey boxes indicate the inability to clone the segment of DNA. Solid boxes indicate the ability to clone the segment of DNA. The question mark refers to a region that can be deleted. It is not known if this is part of *kilA*. Courtesy of Hengen P.N (1991).

A second *kil* region defined as *kilB* was inferred from the inability to construct a plasmid derivative from the Tn5*lac* insertion at coordinate 30.0 (in the derivative pNH142) and the *KpnI* site of pCU1 at coordinate 34.0 (Figure 2). The Tn5*lac* in pNH142 was located by restriction mapping 500 bp counterclockwise of the *kikA*::Tn5 insertion in pCU66. The ability to clone from the Tn5 insertion at coordinate 32.4 (in the derivative pCU171) to the *SaII* site at coordinate 28.8 excludes *kikB* from this region. Thus, *kilB* might be located between coordinate 32.4 and 34.0 on pCU1 and the corresponding *korB* region from coordinate 34.0 (*KpnI* site) to 39.0 (*SaII* site).

The IncN plasmids pKM101 and pCU1 have remarkable similarities in their physical and genetic organization (reviewed by Iyer, 1989). However, it is not known whether the *kil/kor* genes described for the two plasmid are similar. In this work a third *kil* gene was identified in pCU1, it can be cloned in *E. coli* only if a cognate *kor* region is present. In the *Klebsiella* host however, this *kil/kor* region is insufficient for survival and, as will be shown, additional conditions are required.

Killing of *Klebsiella* and *kik* regions

As stated in the Introduction to the thesis, all conjugative plasmids of the group N reduce the viability of *Klebsiella* recipients but not of *E. coli* recipients when mating mixtures are plated on media selective for the recipients and not only the transconjugants. Operationally therefore this host-specific phenotype is

distinguishable from *kil/kor* and was called Kik (*killing of Klebsiella*). In plasmid pCU1, transposon Tn5 mutagenesis and cloning defined two regions that abolish or reduce this phenotype and they were called *kikA* and *kikB* (Figure 1). In the related plasmid pKM101, Winans and Walker (1985a) reported that mutants inactivated in the Kil phenotype still displayed the Kik phenotype. This suggests that these two phenotypes do not have an identical basis. Furthermore and remarkably, *Klebsiella* survivors from a Kik⁺ reaction are genetically immune but do not apparently carry plasmid regions (Gill, 1985). Although the Kik⁺ and Kil⁺ phenotypes of pCU1 and pKM101 are apparently distinct phenotypically and genetically, they may be physiological related. A deeper understanding of the two phenotypes will be necessary to explore such possible relationships. Until then it is useful to retain the separate nomenclature for these operationally distinguishable phenomena and the genes determining them.

***kil/kor* genes in *Streptomyces* plasmids**

Streptomyces are gram-positive soil bacteria that undergo a complex life cycle of morphological differentiation. Numerous extrachromosomal elements have been identified for these organisms that includes plasmids capable of site-specific chromosomal integration (Omer and Cohen, 1989). Most of the plasmids are covalently closed circular DNAs, selftransmissible and nearly all can mobilize

chromosomal markers. Hirochika and Sakaguchi (1982) have also described a linear plasmid in *Streptomyces*.

Plasmid transfer in *Streptomyces* spp is in most of the cases associated with the formation of 'pocks' (lethal zygotis), an area of growth inhibition of the recipient strain. It has been suggested that lethality is a manifestation of transfer and spreading of the plasmid within the mycelium of the recipient cell (Bibb and Hopwood, 1981). Plasmid pIJ101 is the most studied *Streptomyces* plasmid. Its complete sequence (8.9 kb) was deduced by Kendall and Cohen (1988) and the analysis of the plasmid identified four plasmid genes (*kilA*, *spdA*, *spdB* and *kilB*) which are involved in conjugative plasmid transfer and pock formation. Two of them, *kilA* (now known as *tra*) and *kilB* cannot be cloned in *Streptomyces lividans*, on a pIJ101-derived replicon unless the corresponding *kor* functions are provided in *cis* or in *trans* (Kendall and Cohen, 1987). *korA* is able to override the lethal effect of *kilA* and *kilB*, whereas *korB* can only override the lethal effect of *kilB*. The *spdA* and *spdB* genes are responsible for the spreading of the plasmid within the mycelium (Kendall and Cohen, 1988). Deletion of *tra* (formerly *kilA*) prevented the 'pock', while mutations in *kilB* reduces the size of the pock. *kilA* and its regulatory gene *korA* are transcribed from overlapping divergent promoters that are regulated by the *korA* gene product (Stein *et al.*, 1989). The *korB* locus encodes a repressor that regulates its own transcription as well as the transcription from the *kilB* promoter by the binding of KorB to common sequences in each promoter (Zaman

et al., 1992). The footprinting resulting from KorB binding at the *korB* operator/promoter region demonstrated interaction of KorB with the adjacent locus called *sti* (for strong incompatibility), which is involved in pIJ101 replication and incompatibility. This result might suggest a role of KorB in coordinating replication of the plasmid with intramycelial spread during bacterial mating (Tai and Cohen, 1993; 1994).

kil\kor genes have also been described in the plasmid pSAM2, a plasmid from *Streptomyces ambofaciens* and the multicopy plasmid pSN22 from *Streptomyces nigrifaciens* (Kataoka *et al.*, 1991). In pSN22 five genes are involved in transfer and pock formation: *traA*, *traB* and *traR* essential for pock formation and *spdA* and *spdB* which might facilitate the spreading of the plasmid. Mutations in these genes affect pock size. Mutations in *traR* were lethal and deletions in *traB* restored the viability of the *traR* mutation, this result suggests that the lethal product in plasmid pSN22 is the *traB* gene. The excessive expression of *traA* in the absence of *traR* also inhibits cell growth. The authors suggested that the *traR* function of pSN22 is probably the same as that of *korA* in pIJ101. The biological role of these genes is not known, but the observation that plasmid genes that are necessary for plasmid transfer are lethal to the plasmid and /or the host when they are not controlled by the *kor* products suggest that these loci might modulate the transfer process.

KILLING MECHANISMS ASSOCIATED WITH CONJUGATION

Bacterial conjugation is genetically complex and not well understood at the molecular level. There are also clear differences between the processes mediated by plasmids of different transfer groups. The sex pili which seem to play a role in cell to cell contact differ in structure and function (Bradley, 1980). It has been proposed that the base of the pilus may incorporate a membrane spanning channel that allows the DNA transfer (reviewed by Willetts and Skurray, 1987). This has not been demonstrated. In the donor, the plasmid DNA is usually nicked at a specific region, the origin of conjugal transfer (*oriT*) and a single strand is transported in the 5'-3' direction into the recipient cell. It was recently demonstrated for several plasmids that the transferred strand is escorted into the recipient cell by multiples copies of a DNA primase which following transfer initiated DNA synthesis in the recipient on the transferred plasmid (Rees and Wilkins, 1989).

Even though conjugation has been an important factor in the distribution of plasmids between different bacteria, the process itself or the transmission into the recipient have been reported to be harmful. Here I will describe three examples.

LETHAL ZYGOSIS

This lethal effect was reported by Gross (1963) under conditions where an F⁻ bacteria was mated with an excess of Hfr donor cells (Hfr strains contain a F plasmid inserted into the chromosome, the F plasmid in this condition causes the

transfer of chromosomal material to the recipient cell). Under analogous conditions, in $F^+ \times F^-$ matings, the F^- bacteria are not killed (Skurray and Reeves, 1973a). The lethal effect was associated with the inhibition of macromolecular synthesis, failure of some active transport mechanism and leakage of cytoplasmic components to the outside of the recipient cells (Skurray and Reeves, 1973b). Trying to explain why transfer of DNA during conjugation may induce cell killing, Skurray and Reeves (1973b) proposed that during mating, membrane rearrangements may occur in order to transfer the DNA to the interior of the recipient. The prolonged existence of those changes in the membrane in $Hfr \times F^-$ might result in excessive permeability of the F^- recipient and cause the lethal effect. This hypothesis was supported by experiments conducted by Ou (1980), who found that radioactive material was present in the media after mating with Hfr donors and F^- recipients whose membrane were prelabeled with radioactive diaminopimelic acid (DAP). Ou was also able to demonstrate lethal zygosis in $F^+ \times F^-$ matings when large number of donors were used, and the matings were performed in the presence of nalidixic acid. Nalidixic acid is an inhibitor of the enzyme DNA gyrase, and the antibiotic had been shown previously to inhibit the genetic transfer of the F plasmid from a sensitive donor cell (Bouck and Adelberg, 1970).

Bacterial cells which survive the killing effect of lethal zygosis are immune; curing of the F plasmid from the immune cells rendered them subsequently sensitive to lethal zygosis. The region needed for immunity was located by deletion analysis

of several Hfr donor strains in two genes located in the transfer region, *ilzA* (immunity to lethal zygosis) between *traG* and *traS* and *ilzB* close to *traI* (Skurray and Reeves, 1974; 1976). Ou (1980) had suggested that immunity to lethal zygosis could be attributed to a phenomenon called surface or entry exclusion in which a plasmid-carrying population of *E. coli* (F⁺ or Hfr) reduces the acceptance of a second F plasmid by conjugation. He found that surface exclusion (*traS*) mutants were more sensitive to killing by lethal zygosis. The region previously denoted *ilzA* was found to be *traS* one of the two genes that are responsible for the surface exclusion phenotype. This gene encodes an small inner-membrane protein that is believed to block DNA transfer after a mating pair has been established. *ilzB* is most likely *traT*, a transfer gene encoding an outer membrane lipoprotein which acts primarily to inhibit the initial steps in mating-formation (Achtman *et al.*, 1977).

ZYGOTIC INDUCTION

This mechanism of killing described by Jacob and Wollman (1961) is expressed following conjugation between a Hfr donor containing a lysogenic bacteriophage inserted into the chromosome as a prophage (Hayes, 1965). The transfer of the phage genome from the donor to a non-immune (non-lysogenic) recipient will result in the induction of phage development in the recipient, producing the lysis and the release of phage particles. It occurs because the

non-lysogenic recipient lacks the repressor protein which maintains the prophage in an inert state in the donor.

Zygotic induction does not always produce phage particles after the induction. Feinstein and Low (1982) have shown that conjugational transfer of the *rac* (recombination activation) locus of *E. coli* K-12 into a Rac^+ recipient strain produced zygotic induction but no phage particles were shown to be produced under conditions where 98% of the F^- cells were killed. Accumulating evidence suggests that *rac* locus is a cryptic and possibly defective prophage that is excised during the process. Immunity in this case might require the presence of the immunity gene from a similar phage.

KILLING BY EXPRESSION OF *hsd_k* GENES

Type I restriction and modification systems characteristic of *E. coli* K-12 have been studied extensively (for a review see Bickle and Krüger, 1993). The systems comprise three subunits encoded by the genes *hsdR*, *hsdM* and *hsdS*; the product of *hsdS*, is required for recognition of the DNA sequence and together with the product of *hsdM*, is required for modification. The product of the three genes are essential for restriction.

Successful conjugation of wild type *hsd_k* (for *E. coli* K-12 strain host specificity for DNA) genes to non-modified recipient was demonstrated by Colson *et al* (1965). If DNA restriction activity is not controlled, the recipient with no prior

modification should be killed upon transfer and expression of the wild type *hsd_k* genes. Prakash-Cheng *et al* (1993) identified the gene that in *E. coli* C is responsible for the regulation of the *hsd_k* genes in a mutant of *E. coli* C that was killed upon conjugation with the F' plasmid (F101-101) encoding the *hsd_k* genes (*r'_k m'_k*). Killing was demonstrated by the decrease in survival of the recipients in matings with the *r'_k m'_k* donors as compared to the *r_k m_k* donors, when the recipients were selected in media which support the growth of recipients and transconjugants. The killing phenomenon was defined as a *Cre'* phenotype because of the lack of control on the *hsd_k* genes upon transfer, allowing restriction of the non-modified host DNA by the *EcoK* restriction endonuclease. The mutation in *E. coli* C was mapped (by Hfr mapping) between 6 and 16 min on the *E. coli* map. The *Cre'* phenotype presumably controls the expression of the *hsd_k* genes present in the F' plasmid (Prakash-Cheng *et al*, 1993) and in this phenomena the *cre'* allele is acting as the immunity gene. In most of the killing mechanisms presented in this review, control or neutralization of killing (i.e. immunity) is expressed by a gene product (RNA or protein) that is specified by the same plasmid that carries the *kil* genes (although they can also be placed artificially *in trans* to one another on separate plasmids or other compatible vectors). Loss of the plasmid is then accompanied by the loss of immunity. In contrast and as will be shown, the immunity phenomenon that will be described here appears to be unique. It persists in the cell even after the plasmid that gave rise to it is removed.

Materials and Methods

BACTERIAL STRAINS AND PLASMIDS

The bacterial strains and plasmids used in this study, their sources and relevant properties are listed in Tables 1 and 2. In the rest of the thesis, bacterial strains carrying one or more plasmids are indicated by the strains followed by a slash and the designation of the plasmids.

MEDIA, ENZYMES AND CHEMICALS

All strains were routinely grown in modified L Broth (LB) containing: 1% w/v tryptone (Difco), 0.5% w/v NaCl and 0.5% w/v yeast extract (Difco). The same medium was used to prepare agar plates by the addition of 1.5% w/v Difco agar. The minimal media used was that of Davis and Mingioli (1950) which was prepared by mixing pre-sterilized solutions of organic salts (10.5 gm/l K_2HPO_4 , 4.5 gm/l KH_2PO_4 , 1gm/l ammonium acetat , 0.2 gm/l sodium citrate) with sterilized H_2O + agar (1.5% w/v) when the temperature of the media was approximately 50°C. The media were supplemented with sterile solutions of glucose (to 0.2% w/v), $MgSO_4 \cdot 7H_2O$ (to a final concentration of 1mM) and L- histidine (100 µg/ml) for *Klebsiella oxytoca*. If required, the minimal media were also supplemented with Millipore filter-sterilized solutions of amino-acids, vitamins and antibiotics. The latter were added as required: amino acids, 40 µg/ml; vitamin B1 (thiamine) 5 µg/ml; ampicillin 100 µg/ml for *E. coli* strains containing pUC plasmids and 900 µg/ml for *Klebsiella* strains containing pUC plasmids; chloramphenicol 50 µg/ml for

Table 1. List of *E. coli* and *Klebsiella* strains used

Strain	Genotype/ Phenotype	Source or Reference
<i>E. coli</i>		
HB101	F ⁻ <i>hsd20 recA13 ara-14 proA2 lacY1, galK2 rpsL20 xyl-5 mtl-1 λ</i> ⁻	Bolivar <i>et al.</i> , 1977
HB101Rif ^r	Same as HB101 but resistant to 100 µg /ml rifampicin	This laboratory
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21, supE44 λ</i> ⁻	Bachmann, 1972
DH5α	<i>endA1 hsdR17 supE44 thi-1 recA1, gyrA relA1 λ</i> ⁻	Grant, 1990
S17-1	<i>pro chr::RP4</i>	Simon <i>et al.</i> , 1983
S17-1 <i>λpir</i> ⁺	<i>pro chr::RP4 λpir</i>	"
<i>Klebsiella oxytoca</i>		
M5a1	Prototroph, Ap ^r	MacNeil <i>et al.</i> , 1978
M8	Same as M5a1	Rodríguez, 1981; M5a1/pCU1, cured of plasmid pCU1

Table 1. continued

UN	prototroph, same as M5a1	MacNeil <i>et al.</i> , 1981
UN2979	<i>hisD4226 lacZ4001</i> galactose resistant	"
UN2979-Rif ^r	Same as UN2979 but resistant to rifampicin	"
UNC109	Same as UN2979	This study, UN2979/pCU109 cured of plasmid pCU109
UNC1139	Same as UN2979	This study, UN2979/pCU1139 cured of plasmid pCU1139
UNC403	Same as UN2979	This study, UN2979/pCU403 cured of plasmid pCU403
UNC403-1	Same as UN2979	This study, UN2979/pCU403-1 cured of plasmid pCU403-1
UNC56-13	Same as UN2979	This study, UN2979/pCU56-13 cured of plasmid pCU56-13
UNCG645	Same as UN2979	This study, UN2979/pAAG645 cured of plasmid pAAG645
UNCG445	Same as UN2979	This study, UN2979/pAAG445 cured of plasmid pAAG445
UNCG345	Same as UN2979	This study, UN2979/pAAG345 cured of plasmid pAAG345
UN2979::400 Km ^r	Same as UN2979, with a mini Tn10 containing 400 bp of pUC DNA	This study

Table 2. List of plasmid used

Plasmid	Marker(s)	Description	Source or Reference
pACYC184	Cm,Tc	cloning vector	Chang and Cohen, 1978
pUC128	Ap	cloning vector	Keen <i>et al.</i> , 1988
pNH Kan/ <i>oriT</i>	Ap, Km	cloning vector containing <i>oriT</i> _{RK2}	Hengen and Iyer, 1992b
pCU1	Ap, Sm, Sp	parental naturally- occurring plasmid	Konarska-Kozłowska and Iyer, 1981
pCU88	Ap, Sm., Sp, Km	Tn5 insertion in pCU1 at coordinate 10.2	Thatte and Iyer, 1983
pCU109	Cm	pCU1 <i>tra</i> region Coordinates 27.7 to 10.2 cloned in pACYC184	Thatte and Iyer, 1983
pCU812	Cm, Km	Tn5 insertion from pCU171 in pCU109 at coordinate 32.4	Rotheim <i>et al.</i> , 1988
pVT80	Cm, Km	Tn5 insertion in pCU109 at coordinate 32.8	Thatte <i>et al.</i> , 1985a
pVT70	Cm, Km	Tn5 insertion in pCU109 at coordinate 33.5	"
pVT84	Cm, Km	Tn5 insertion in pCU109 at coordinate 33.9	"

Table 2. continued

pVT83	Cm, Km	Tn5 insertion in pCU109 at coordinate 34.2	Thatte <i>et al.</i> , 1985a
pVT71	Cm, Km	Tn5 insertion in pCU109 at coordinate 36.4	"
pVT96	Cm, Km	Tn5 insertion in pCU109 at coordinate 38.0	"
pVT82	Cm, Km	Tn5 insertion in pCU109 at coordinate 0.7	"
pVT61	Cm, Km	Tn5 insertion in pCU109 at coordinate 2.5	"
pVT213	Cm, Km	Tn5 insertion in pCU109 at coordinate 3.1	"
pVT149	Cm, Km	Tn5 insertion in pCU109 at coordinate 6.5	"
pVT149-1	Cm	<i>Xho</i> I deletion of pVT149	This study
pCU29	Ap, Sm, Km	Tn5 insertion in pCU1 at coordinate 8.7	Thatte and Iyer, 1983
pCU101	Cm	pCU1 <i>tra</i> region from coordinate 27.7 to 8.7 cloned in pACYC184	"

Table 2. continued

pCU105	Cm	pCU1 DNA (coordinate 27.7 to 9.5) cloned in pACYC184	Thatte <i>et al.</i> , 1985b
pCU51	Km	deletion derivative of pCU29	"
pCU56	Cm	<i>oriT</i> region of pCU1 cloned in pACYC184	Thatte <i>et al.</i> , 1985a
pCU66	Ap, Sm, Sp, Km	Tn5 insertion in pCU1 at coordinate 30.2	Thatte <i>et al.</i> , 1985b
pCU403	Km	<i>Bam</i> HI deletion derivative of pCU66	"
pCU403-1	Km	<i>Kpn</i> I deletion of pCU403	This study
pAAG13	Km, Ap	6.9 <i>Bam</i> HI- <i>Kpn</i> I fragment from pCU403-1 cloned in pUC128	"
pCU56-13	Cm	5.9 <i>Sph</i> I fragment from pAAG13 cloned in pUC128	"
pCU1107	Cm	<i>kikA</i> region from pCU171 cloned in pACYC184	Hengen, 1991
pCU1139	Cm, Km	Kan/ <i>oriT</i> _{KK2} cloned in pCU1107	"
pCU1140	Cm, Km	Kan/ <i>oriT</i> _{KK2} cloned in pCU1107	"

Table 2. continued

pCU1140-A	Cm, Km	Imm ⁺ derivative from pCU1140	This study
pCU11402	Cm, Km	Imm ⁺ derivative from pCU1140	"
pCU11403	Cm, Km	insertion of IS10-like in pCU1140	"
pCU11404	Cm, Km	insertion of extra DNA in pCU1140	"
pAAG8	Ap	Bal31 deletion of pCU1107 cloned in pUC128	"
pAAG845	Ap, Km	Kan/ <i>oriT</i> _{KK2} cloned in pAAG8	"
pAAG8451	Ap, Km	insertion of IS1-like element in pAAG845	"
pAAG7	Ap	Bal31 deletion of pAAG8	"
pAAG745	Ap, Km	Kan/ <i>oriT</i> _{KK2} cloned in pAAG7	"
pAAG7451	Ap, Km	Imm ⁺ mutant of pAAG745	"
pAAG6	Ap	Bal31 deletion of pAAG8	"
pAAG645	Ap, Km	Kan/ <i>oriT</i> _{KK2} cloned in pAAG6	"

Table 2. continued

pAAG5	Ap	<i>Hind</i> III- <i>Pst</i> I fragment of pAAG6 cloned in pUC128	This study
pAAG5Ω	Ap, Sm, Sp	Ω fragment inserted at <i>Hind</i> III site of pAAG5	"
pAAG4	Ap	<i>Bal</i> 31 deletion of pAAG5	
pAAG445	Ap, Km	Kan/ <i>oriT</i> _{RK2} cloned in pAAG4	"
pAAG3	Ap	<i>Bal</i> 31 deletion of pAAG5	"
pAAG345	Ap, Km	Kan/ <i>oriT</i> _{RK2} cloned in pAAG3	"
pAAG2	Ap	<i>Bal</i> 31 deletion of pAAG5	"
pAAG245	Ap, Km	Kan/ <i>oriT</i> _{RK2} cloned in pAAG2	"
pAAG10	Ap	<i>Ssp</i> I deletion of pAAG5	"
pAAG15	Ap	<i>Nar</i> I deletion of pAAG5	"
pAAG15-13	Ap	<i>Bal</i> 31 deletion of pAAG15	"
pAAG1	Ap	<i>Pst</i> I- <i>Hinc</i> II deletion of pAAG5	"

Table 2. continued

pAAG1-184	Cm	<i>Bam</i> HI- <i>Hind</i> III fragment of pAAG1 cloned in pACYC184	This study
pAAG100	Ap	mutant of pAAG1	"
pAAG103	Cm	<i>Ssp</i> I- <i>Hind</i> III fragment of pAAG1 cloned in pACYC184	"
pFSG1	Ap	<i>Hpa</i> I fragment from pCU109 (<i>kikB</i> region) cloned into pUC128	This study
pLOF/Km	Km	Cloning vector	Herrero <i>et al.</i> , 1990
pUC18Sfi	Ap	Cloning vector	"
pMRK400	Km	400 bp of pUC18Sfi cloned in pLOF/Km	This study
pPH4	Ap	<i>kikA</i> fragment in pJF118HE	Hengen <i>et al.</i> , 1992a

E. coli strains and 100 µg/ml for *Klebsiella oxytoca* strains; tetracycline 15 µg/ml; kanamycin sulphate, 50 µg/ml; rifampicin 100 µg/ml; nalidixic acid 50 µg/ml; streptomycin 50 µg/ml; spectomycin 50 µg/ml and 100 µg/ml when *Klebsiella* was used. The antibiotics were purchased from Commercial Sources. They were dissolved in sterile distilled water at appropriate concentrations and stored at 4°C. Rifampicin was prepared fresh before use in methanol as the solvent.

Restriction enzymes and linkers were purchased from New England Biolab Ltd, Mississauga, Ontario. T4 DNA ligase, Klenow enzyme and 1kb MW standards of DNA were purchased from GIBCO-BRL Life Technologies Ltd., Burlington, Ont. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was obtained from Boehringer-Mannheim Canada Ltd. Deoxyadenosine 5'-triphosphate, tetra (triethylammonium) salt, [α-³²P] (3000 Ci/mol specific activity) and deoxyadenosine 5'-[α-thio] triphosphate, [α-³⁵S] were purchased from Amersham Canada Ltd, Oakville, Ont. Other fine chemicals were from Sigma Chemical Corp., USA.

BACTERIAL GROWTH CONDITIONS

All strains were routinely grown in LB, aerated by agitation in a shaker overnight at 37°C. Logarithmic cultures were prepared by diluting the overnight culture 100 fold and regrowing them for 2½ to 3 h to give a viable cell count of approximately 2 to 3x 10⁸ cell/ml. When stationary recipients were used in matings

they were diluted 20 fold from the overnight culture and incubated at 37°C in the shaker until maximum turbidity was reached.

For purification of *Klebsiella oxytoca* transconjugants, isolated colonies were streaked on the selective media and after two rounds of 'single-colony purification' they were inoculated in liquid minimal media with the required antibiotic.

Stock cultures were maintained at -20 and -80°C in LB containing 20% (v/v) of glycerol (Miller, 1972). Strains were stored for short terms on slants or plates of appropriate media at room temperature and at 4°C respectively.

BACTERIAL CONJUGATION

Filter mating. This procedure was used for construction of strains and for determining the frequency of transfer of a plasmid from an R⁺ strain. An overnight culture of an R⁺ strain grown in LB containing antibiotic was diluted 1:100 in fresh media and grown for 3 hours until the culture reached mid-log (approximately 2×10^8 cell/ml). 0.2 ml of the donor culture was mixed with 0.2 ml of a stationary culture of the recipients cells. The mixture was collected on a millipore membrane (47 mm, 0.45 µm pore) and placed on a prewarmed LB plate, and the plate was incubated for 1 hour at 37°C. At the end of the incubation, the cells were washed from the filter by vigorous vortexing in 2 ml of saline (0.85% w/v NaCl). Serial dilutions were prepared in saline and were plated on media selective for transconjugants. Donor and recipient (0.1ml) were plated separately as controls on

the same media. The plates were incubated for 24 to 48 hours (depending upon the media used). After two step purification on selective media, transconjugants were analyzed further for their plasmid-associated phenotypes and the plasmid DNA was isolated from them to examine their restriction pattern on agarose gels.

KILLING AND IMMUNITY TO KILLING

A qualitative test to check the ability of a donor cell to kill *Klebsiella* was performed by spotting a multiply auxotrophic exponentially growing culture of the donor onto a lawn of diluted (10x) overnight *Klebsiella* culture placed on minimal media plates, a clearing in the region where the donor was spotted was an indication of a positive result. The spot test when used for screening a large number of transconjugants was performed using a multipoint inoculation device that allowed the analysis of 25 colonies per plate. Cells from single colonies were transferred by a sterile toothpick to a fresh selective agar media in an ordered fashion and regrown for 24 hours at 37°C. Using a sterile twenty five multipoint inoculator, a sample of the colonies were transferred to a 25 compartment sterile culture container (Elisa, Milano, Italy) containing in each compartment 0.5 ml of saline. The multipoint inoculator was then used to transfer the 25 samples onto a glucose minimal plate. The samples on the glucose minimal media were allowed to air-dry and then spotted with aliquots of an exponentially growing culture of the donors cells using a second multipoint device (of lesser diameter than the first) which delivered approximately

5µl of the donor culture onto the dried drop of inoculated transconjugants. Figure 2, shows an example of the results of the test. The analysis of *Klebsiella* cells for immunity was done using this test. Imm⁻ derivatives gave a zone of inhibition (see Figure 2 left), Imm⁺ derivatives are protected and there is not a zone of inhibition (see Figure 2, centre) a result comparable to the control experiment where only the *E. coli* R⁻ strain is spotted (Figure 2, right).

CONSTRUCTION OF A CHROMOSOMAL INSERTION BY THE SUICIDAL VECTOR pLOFKm

A 400 bp fragment from pUC18Sfi (Herrero *et al.*, 1990) was inserted into the *Klebsiella* chromosome as illustrated in Figure 4. The 400 bp fragment from pUC18Sfi was inserted into the suicide transposon donor plasmid pLOF/Km, which carries a Km indicator gene and the transposition system of Tn10-IS10_R with only the inverted repeats and the transposase gene regulated by *ptac* promoter (Herrero *et al.*, 1990). The donor plasmid contains the origin of replication of R6K and the conjugal transfer origin (*oriT*) of RP4 plasmid. This construct could be maintained when provide in trans with the R6K-specified π protein from the R6K *pir* gene, placed in the *E. coli* chromosome. For this I used the strain S17-1(λ pir) which contains the RP4 plasmid and the *pir* gene of R6K inserted into the chromosome and can be used as a conjugative helper system. The construct containing the target DNA into the mini-transposon of pLOFKm was denoted pMRK400, it was transformed

Figure 3. Illustration of the results of the 'spot test' used for the detection of the Kik phenotype (killing of *Klebsiella*). The test was used to isolate Kik⁻ mutants of the plasmid or immune derivatives of *Klebsiella*. The drop on the left indicates killing of Imm⁻ *Klebsiella* by a drop of C600/pCU1 culture (R⁺ strain), small colonies growing inside the killing spot are survivors that carry the plasmid and have an Imm⁺ phenotype. The drop in the middle is of an Imm⁻ *Klebsiella* spotted with a R⁻ strain. The drop on the right represents the result of an Imm⁺ *Klebsiella* spotted with a drop of the C600/ pCU1 culture.

into the helper strain S17-1(λ *pir*) and one of the Km resistant transformants was then used as donor in *Klebsiella* matings. *Klebsiella* does not contain the *pir* product to allow the replication of the plasmid; therefore, kanamycin resistant colonies arising from a mating with S17-1 λ *pir*/pMRK400 might be the product of a transposition event with the insertion of the foreign DNA (400bp) into the chromosome. The purpose of this construct was to provide a positive control for DNA hybridization experiments with *Klebsiella* chromosomal DNA.

EXTRACTION OF PLASMID DNA

1. SMALL SCALE PREPARATION OF PLASMID DNA

The boiling procedure of Holmes and Quigley (1981) was used to extract plasmid DNA for the rapid analyses of recombinants. The bacterial strain carrying the plasmid was grown overnight in 5 ml of LB supplemented with an appropriate antibiotic for selection. The culture was cooled on ice for 5 min and the cells were collected by centrifugation at 6,000xg in a table top centrifuge for 10 min. The cell pellet was resuspended in 400 μ l of cold STET (8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM of Tris-HCl pH 8.0) and transferred to a microfuge tube containing 25 μ l of freshly prepared lysozyme (Sigma, 2X crystallized lysozyme) solution, 1mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The tube was placed immediately in a boiling water bath for 50 seconds. The lysate was spun in an Eppendorf centrifuge at 40,000xg for 15 min and the gelatinous pellet was

Figure 4. Outline of the strategy employed to insert a 400 bp fragment into the *Klebsiella* chromosome. The fragment from pUC18Sfi was inserted into the *Sfi* site in the delivery vector pLOF/Km (Herrero *et al.*, 1990) to give the conditionally suicide vector pMRK400. This was transformed into the Pir providing strain S17-1 λ *pir* from which it was mated into the strain UN2979 selecting for Km^r transconjugants that have the modified transposon containing the 400 bp inserted into the chromosome.

Transformation

S17-1 λ *pir* / pMRK400

Conjugation

UN2979 with
400 bp insert

removed with a sterile toothpick. The DNA was precipitated from the clear supernatant with 0.25 volume of 10 M ammonium acetate and 2 volumes of cold absolute ethanol at -20°C for 60 min. After centrifugation at 40,000xg for 30 min, the precipitated DNA was washed in 70% cold ethanol. The pellet was dried in a vacuum oven for 60 min and was dissolved in 25 µl of TE buffer.

2. LARGE SCALE PREPARATION OF PLASMID DNA

The isolation of large amounts of plasmid DNA was by the cleared lysate procedure described by Clewell and Helinski (1969) with the modification described by Humphreys *et al* (1975). 10 ml of an overnight culture of the desired strain grown in the presence of antibiotic was diluted to 1 litre in fresh sterile medium with antibiotic. The culture was grown to stationary phase and the cells were pelleted by centrifugation at 10,000xg for 10 minutes at 0°-5°C in a GSA rotor. The cells were resuspended in 50 ml of cold 25% Sucrose, 0.1 M Tris-HCl (pH 8.0) on ice. 6 ml of a freshly prepared lysozyme solution, (1mg/ml in TE) was added to the cell suspension and maintained for 5 minutes on ice; then, 28 ml of 0.25 M EDTA pH 8.0 was added. The suspension was kept at 0°C (swirling occasionally) for another 5 minutes after which lysis was brought about by the addition of 50 ml of a detergent mixture (1% Brij-58, 0.4% sodium deoxycholate, 0.0625 M EDTA and 0.05 M Tris-HCl pH 8.0). After 5 minutes, the sample became relatively clear and viscous; it was then centrifuged at 4°C for 25 minutes at 35,000xg in an SS-34 rotor;

this step pelleted the cell debris and most of the chromosomal DNA, while the plasmid DNA was recovered in the supernatant. To this supernatant called "cleared lysate", NaCl was added to a final concentration of 0.5 M and polyethylene glycol (PEG 6,000) to a concentration of 10% (w/v). These were dissolved by gentle swirling and the solution stored overnight at 4°C. The precipitate that was formed was collected by centrifugation in a SS-34 rotor at 8,000xg for 3 minutes and was dissolved in the minimal volume of TE buffer required. Ethidium bromide (10mg/ml stock solution in TE buffer) was added to the preparation to a final concentration of 500 µg/ml and solid CsCl was added until the solution reached a refractive index of 1.380 - 1.385 (as determined by using a Bausch and Lomb refractometer). The sample was centrifuged in a Beckman VTi 80 rotor using a Beckman L8-80 ultracentrifuge at 270,000xg overnight at 15°C. After centrifugation, the plasmid DNA band that was separated from the chromosomal DNA band was recovered by puncturing the tube with a 1 ml disposable hypodermic syringe and withdrawing the plasmid band under conditions of illumination with a long wave ultraviolet lamp.

Ethidium bromide was extracted from the DNA solution by adding CsCl-saturated isopropanol and discarding the alcohol layer which contained the ethidium bromide; this step was repeated several times until all visible traces of the dye were removed. To the resulting plasmid DNA solution (1 ml), the same volume of H₂O was added followed by 2 volumes of ethyl alcohol, mixed and stored overnight at room temperature. The DNA was precipitated by centrifugation in an SS-34 rotor at

10,000 xg for 10 minutes; the precipitation process was repeated to ensure the complete removal of CsCl. The precipitated DNA was finally dissolved in TE buffer. The approximate concentration of DNA in the sample was determined by measuring the optical density at 260nm of a known dilution of the sample in the buffer, an O.D of 1.0 being equivalent to 50 µg/ml. (Maniatis *et al.*, 1982).

ISOLATION OF TOTAL BACTERIAL DNA

Chromosomal DNA was isolated by the method described by Meade *et al* (1982) with some modifications. 5 ml of overnight culture was pelleted and resuspended in cold TES buffer (10 mM Tris-HCl, 1mM EDTA pH 8.0) containing 100 mM NaCl. The cells were pelleted and resuspended in 5 ml of cold TE buffer (10 mM Tris-HCl and 1mM EDTA pH 8.0), 0.5 ml of fresh prepared lysozyme (2 mg/ml in TE) was added and the mixture was incubated at room temperature for 20 min. The cells were lysed by 0.6 ml of 10% SDS and proteinase K (200 µg/ml) added and the mixture was incubated at 37°C for 1 hour. The DNA was extracted twice with an equal volume of phenol (saturated with 0.01 M Tris, pH 8.0) and then with 5 ml of chloroform. The aqueous phase was brought to 0.3 M ammonium acetate and the DNA was precipitated by adding two volumes of cold ethanol. The precipitated DNA was removed with a glass rod, transfer to a Eppendorf tube then washed with 70% ethanol, air dried and dissolved in sterile TE buffer.

CURING OF PLASMIDS FROM KLEBSIELLA

Spontaneously cured derivatives were isolated by subculturing the plasmid containing strain in antibiotic-free media for several generations. This procedure was preferred to avoid the use of one of several agents like ethidium bromide which have been used in curing plasmids but could also be causing mutations. After two days, the culture was diluted and plated in LB-agar plates. Individual colonies were replicated in LB-agar with and without the corresponding antibiotic. The colonies that did not grow in LB-agar with the antibiotic were inferred to be cured of the plasmid. This was confirmed by isolating total DNA by the boiling method and analyzing it by gel electrophoresis.

RESTRICTION ENDONUCLEASE DIGESTION OF DNA

The restriction endonuclease digestion of plasmid DNA was performed with 1-2 µg of DNA using 1 µl of the 10x reaction buffer (as recommended by the supplier), 1 µl of 10x BSA solution, sufficient amount of the appropriate restriction enzyme (to ensure complete digestion) and sterile distilled water to bring the final volume to 10 µl. The reaction mixture was incubated for one to two hours at the recommended temperature for each specific restriction enzyme. When the DNA was isolated from mini-preps the digestion was performed in the presence of RNase solution (10 mg /ml). After the digestion was completed, 0.1 volume of the stop solution (loading buffer, containing 0.25% w/v bromophenol blue, 100 mM EDTA

pH 8.0 and 40% w/v glycerol) was added before loading the mixture on an agarose gel for electrophoresis.

Digestion of genomic DNA was performed using 5 to 10 µg of chromosomal DNA in 50 µl reaction with an excess of the restriction enzyme. The samples were incubated at an optimum temperature overnight.

ANALYSIS OF DNA BY AGAROSE GEL ELECTROPHORESIS

Fragments of restricted DNA were separated by agarose gel electrophoresis in a 0.8% to 1% agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate and 2 mM EDTA pH 8.0). The gel was run in a horizontal, submerged, mini cell electrophoresis unit at 100V for 60 min. Genomic DNA fragments were separated in a larger electrophoresis unit at 10V overnight. The current for electrophoresis was supplied by a power supply EPS, Pharmacia Fine Chemicals. After electrophoresis the gel was stained with Ethidium bromide (0.5 µg/ml) and destained in distilled water. Gels were illuminated by a Fotodyne Foto/prep I transilluminator and photographed using a Polaroid MP4 vertical camera using a red filter and using type 57 or 55 polaroid film. The DNA size standard used was BRL 1 kb DNA ladder with fragments size in base pair (s) (bp) of : 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, and 517/ 506. DNA of bacteriophage Lambda digested with the enzyme *HindIII* was also used as molecular standard with fragments size in bp of : 23130, 9416, 6557, 4361, 2322,

2027, 564 and 125.

The computer program DNA Frag (Schaffer and Sederoff, 1981) was used to calculate the fragment size of digested DNA.

RECOVERY OF DNA FROM AGAROSE GELS

The DNA was extracted from agarose gels after the sample was digested and electrophoresed in TAE buffer and illuminated under longwave UV lamp. The gel fragment containing the band of interest was sliced out of the gel using a razor blade and transferred to a clean microfuge tube. For small molecular weight fragments the DNA was recovered using glass-milk and the GeneClean kit purchased from Bio101 Inc, La Jolla CA., following the protocol recommended by the manufacturer.

For DNA fragments bigger than 10 kb the recovery of the DNA from the agarose gel was done by the Electroelution method. Briefly, the gel slice with the DNA fragment was transferred to a dialysis bag containing electrophoresis buffer and the bag was sealed after expelling the air bubbles. The dialysis bag was placed in the gel tank submerged and arranged so that the DNA could come out of the agarose by the shortest route. Electrophoresis was performed for one hour and after, the DNA was collected from the buffer by adding 1/5 of the volume of 10 M ammonium acetate and 2 volume of ethanol for precipitation.

LIGATION OF DNA

Ligation of restricted DNA to construct recombinant molecules was done using T4 DNA ligase in a 10 µl reaction, using 2 µl of the 5x buffer supplied by the manufacturer. The ligations were incubated overnight at 15°C and 5 µl was used to transform the appropriate competent cell. When linkers were added to the molecule the ligation was performed on DNA whose free end were repaired by the Klenow enzyme using a mixture of the four dNTPs in the appropriate buffer. The fill-in reaction was stopped by heating the enzyme at 70°C for 20 min. This DNA was suitable for the addition of linkers by T4 ligase as explained before. After ligation, the excess of concatemer linkers were removed by digesting the reaction with an excess of the restriction enzyme. The DNA was isolated using 'glass milk' (Gene clean) before recircularization of the molecule by T4 ligase.

DELETION OF DNA SEQUENCES UTILIZING EXONUCLEASE *BAL31*.

Bal31 exonuclease (BRL) was used to generate deletions in plasmid DNA. The DNA was first linearized with an appropriate restriction enzyme; after the digestion, the DNA was isolated with 'glass milk' using the GeneClean procedure. The DNA was resuspended in sterile water, 4 µl of 10x *Bal31* buffer (200 mM Tris-HCl pH 8.1, 125 mM MgSO₄, 125 mM MgCl₂, 6M NaCl, 10 mM EDTA) and 1 µl of the enzyme *Bal31* (1-2 units) were added to a final volume of 40 µl reaction.

The mixture was incubated at 37°C. After varying periods of time, aliquots of the reaction mixture were removed and the reaction was stopped by adding EGTA to a final concentration of 20 mM. The DNA was purified from the mixture using glass milk. The staggered ends of the *Bal*31 treated DNA were repaired by filling in the ends with a mixture of the four dNTPs (3 µl of a 2 mM stock) using 1 unit/µl of the Klenow fragment of *E. coli* polymerase I enzyme in 20 µl reaction containing 2µl of the 10x fill-in buffer (500 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 10 mM DTT and 500 µg/ml of bovine serum albumin). The reaction was incubated at 37°C for twenty min and the enzyme was inactivated by heat after the fill-in reaction. The blunt-end DNA was ligated to linkers to generate a new restriction site or ligated directly to circularized the molecule. When linkers were added the process was followed by two ligation reactions, the first was the ligation of the linker to the DNA molecule and since linkers can be ligated to form long blunt-ended concatemers it was necessary to generate cohesive ends by cleaving with the required restriction enzyme in order to get rid of the excess of linkers before the second ligation, which created the cohesive ends.

BACTERIAL TRANSFORMATION

Cells were made 'competent' according to the procedure of Cohen *et al* (1972) with slight modifications. An overnight culture of the appropriate strain

grown in LB was diluted 100-fold and regrown to an optical density of 0.6- 0.8. The cells were chilled on ice for 20 min and sedimented at 8000xg for 10 min at 4°C in a bench top centrifuge. The cell pellet was washed twice, first with 1 volume of 100 mM cold MgCl₂ and the second wash with 1 volume of 100 mM CaCl₂. After the final wash the cells were resuspended in 1/10 of the original volume of 100 mM CaCl₂ solution. 100 µl of this preparation was used for transformation with 100-200 ng of plasmid DNA. The transformation mixture was incubated on ice for 30 min, heat pulsed at 37°C for 5 min, chilled on ice for five min, diluted 10 fold with LB and incubated for 1 hour at 37°C before plating on selective media.

When pUC plasmids were used for cloning with the *E. coli* strain DH5α, the selection of transformants was done on selective plates containing 100 µg/ml X-gal dissolved in dimethyl-formamide. The recombinant transformants were identified by their ability to form colourless colonies on X-gal medium while non recombinants produced blue colonies.

SOUTHERN BLOTS, LABELLING OF PROBES AND HYBRIDIZATION

Genomic DNA from Imm⁻ and Imm⁺ *Klebsiella* strains was restricted with the enzyme *Hind*III and separated by agarose gel electrophoresis. The genomic DNA fragments were transferred to a Hybond-N nylon membrane (Amersham Canada Ltd.) by capillary transfer (Southern, 1975) as described in Maniatis (1982). The gel was stained with ethidium bromide and photographed as described above.

The gel was soaked in 0.25 M HCl for 10 min to fragment the DNA, rinsed with H₂O to remove the acid, transferred to a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min and after to a neutralizing solution (1.5 M NaCl, 0.5 Tris-HCl, pH 7.4) for 30 min with gentle agitation. The gel was placed on a plexiglass tray over a 3MM paper with the ends immersed in the 20x SSC blotting buffer (3 M NaCl; 0.3 M sodium citrate) and it was covered with a piece of Hybond-N Nylon membrane, two pieces of 3MM paper (of the same size of the gel) saturated with the blotting buffer and followed by 2 cm stack of paper towels and a light weight. After transfer, the filter was removed and soaked in 2x SSC (0.3 M NaCl, 30 mM sodium citrate) in order to remove any traces of agarose. The filter was air-dried for 15 min and the DNA was bound to the nylon filter by exposure to UV light for 5 min in a transilluminator.

The membrane was placed in a glass tube with 10 ml of the hybridization solution. The composition of this solution was as follows: 50% formamide (Sigma); 6x SSC; 5x Denhardt's reagent; 0.5% SDS and 250 µg/ml of denatured salmon sperm DNA (Sigma). The tube was placed in a hybridization incubator (Model 310, Robbins Scientific) at 45°C for four h.

Labelling of plasmid DNA fragments or the plasmid to be used as probes was by nick translation (Rigby *et al.*, 1977) as described by Maniatis *et al* (1982) using 0.5 µg of DNA, 25 µCi of radiolabelled deoxyadenosine (α -³²P) triphosphate (Amersham), 5 µl of the nucleotide solution and 5 µl of the DNase/DNA polymerase I

solution purchased from Bethesda Research Labs. Inc. (BRL), in a final volume of 50 μ l reaction. The reaction mixture was incubated at 15°C for 1 h and the reaction was stopped by the addition of 5 μ l of 0.25 mM EDTA. The unincorporated nucleotides were removed by gel filtration. The sample was loaded in a Pasteur pipette column containing Sephadex G-50 (Pharmacia) which was pre-equilibrated with 5x SSC buffer. This buffer was also used to elute the DNA from the column. Aliquots of 200 μ l were collected in microfuge tubes. The sample in the tubes containing the peak of incorporation were collected and boiled for 10 min in order to denature the DNA. The tube was placed on ice until the sample was added to the hybridization tube.

DNA Hybridization was performed in 10 ml of fresh pre-warmed hybridization buffer and the denatured probe, overnight at 42°C. After hybridization the membrane was washed two times with 50 ml portions of 2x SSC, 0.1% SDS for 5 min at room temperature, twice with 0.5x SSC, 0.1% SDS and then twice with 50 ml portions of 0.1x SSC, 0.1% SDS for 10 additional min each at 45°C. The membrane was allowed to air dry and was then placed inside a cassette with a DuPont intensifying screen and exposed to a Kodak-X-OMAT AR film at -70°C overnight to two days. Autoradiograms were developed using an AFP Imaging 14XI. X-ray film processor. The developer and fixer were purchased from Picker Chemicals and were used as recommended by the manufacturer.

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PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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PRECISIONSM RESOLUTION TARGETS

DNA SEQUENCING

DNA sequencing was done by the dideoxy method of Sanger *et al* (1977) with the modification proposed by Disotell *et al* (1992). The Sequenase Version 2.0 was purchased from United States Biochemical Corporation (USB), Cleveland, OH. The reagents were mixed as follows: 2 μ l of 5x sequencing buffer and 1 μ l of a 20 pmol/ μ l of primer were added to 7 μ l of purified plasmid DNA. The mixture was placed at 100°C for 5 min, then "snap-cooled" by immediately immersing the tube in a ice-water bath. A labelling "cocktail" containing 2 μ l of (1: 5 dilution) labelling mixture, 1 μ l of 100 mM dithiothreitol (DTT), 0.5 μ l of [α ³⁵ S]-d ATP and 2 μ l of dilute Sequenase was added to the tube containing the DNA-primer mixture and incubated for one minute at room temperature. A volume of 3.2 μ l was transferred to the appropriated terminating mixture and incubated for 5 minutes at 42°C. To eliminate ambiguities in several regions caused by band compression the dGTP labelling mixture was substituted by the dITP labelling mixture and the dGTP termination mixture were substituted by the dITP termination mixtures. The reaction was stopped by the addition of 4 μ l of the stop solution provided with the kit and denatured in a boiling water before being fractionated on a polyacrylamide gel.

The BRL sequencing gel unit MS2 and vertical 7.5 % w/v polyacrylamide gels were used to fractionate DNA. It was prepared with 15 ml of 38 % acrylamide, 2 % NN'- bis-acrylamide (bis) stock solution mixed with 20 ml of 5x TBE buffer (1x TBE is 89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA pH 8.2), 42 g

ultrapure urea (Sigma), 1 ml w/v ammonium persulfate, 60 µl of Temed and water to a final volume of 100 ml. The gel was cast between 30 x 34 cm glass plates using 0.3 mm side-spacers, the gel was poured and allowed to polymerize overnight. Home prepared combs were used to generate the wells. The gels were prerun for 60 min at 1000V by connecting the unit to a BioRad power supplied 3000/300. After the samples were loaded the gel was run at 1300-1500V until the bromophenol blue ran off the gel. After electrophoresis, the gel was fixed in a 10% v/v acetic acid; 10% v/v methanol solution for 30 min, transferred to a 3MM Whatman paper and dried in a gel dryer unit under vacuum at 80°C for 60 min. X-Omat AR film was exposed 24-48 h at room temperature and developed in a film processor as described before.

COMPUTER ASSISTED DNA SEQUENCE ANALYSIS

Analysis of nucleotide sequences, prediction of open reading frames, translation products and calculation of molecular weight were performed using an IBM compatible software package 'Microgenie' (Queen and Korn, 1984) sold by Beckman Instruments Inc., Palo Alto, California, U.S.A and the Program MacDNASIS Pro. V3.0, Hitachi Software Engineering Co. 1993. Data base searches for DNA and amino acid were done by using the NCBI BLAST network service (Altschul *et al.*, 1990) and FASTA (EMBL network service) (Pearson and Lipman, 1988).

Table 3. List of oligonucleotide primers used

Designation	DNA sequence	Source
MR1	5'-CATACTTCTTAAACAACGTGT	Queen's University
MR2	5'-TCTGTGATTGCAATACAAGAT	"
MR3	5'-TAGGGATTTTCAAATCAG	"
MR4	5'-TTTAACTGAATACTACCG	"
MR5	5'-GGGCTAATGAGATCGTG	"
MR6	5'-CCACATGGCTCAAAGAGC	"
KIKA	5'-TATCGCCTGCAATATATTCG	"
MI-1	5'-ATTGCCGGTTAAATCTGC	"
MI-2	5'-GAGGTAACGATAGAACGG	"
MI-3	5'-TGCTACTGACGGCCAGAC	"
MI-4	5'-GTATTCAGTTAAAACAGC	
MI-5	5'-TCTGATTTTCAGGGGAACC	Carleton University
MI-6	5'-GATCATCAAGTGTTAAAC	"
Tn5-30	5'-AACGGGAAAGGTTCCGTT	Queen's University
M13 reverse	5'-TAACAATTTACACAGG	"
M13 forward	5'-CCCAGTCACGACGTTGT	"
p51 -B	5'-ACTGGCCCGCCGTTATAC	Carleton University
p51-C	5'-TTAGGAGACACGGCAGAC	"
p51-D	5'-TATTACAGCTGGCTGCAC	"
p51-M	5'-TTCCTCCACGTGACTGTC	"

RESULTS AND DISCUSSION

Results Part 1

**Identification and properties of a plasmid region needed
for the establishment of immunity**

Immunity acquired by mating with *E. coli* carrying pCU1 is an inherited property that does not require the continued presence of the plasmid.

Klebsiella strains that survive and carry pCU1 are immune to the Kik' phenotype when they are used as recipients in a second mating with *E. coli* C600 (pCU1). It was considered possible that the basis of such superinfection immunity was due to a phenomena such as entry exclusion or intracellular incompatibility that is generally exhibited by a resident conjugative plasmid toward an incoming plasmid of the same group (Couturier *et al.*, 1988). However, Gill (1985) derived spontaneous derivatives of *Klebsiella* carrying pCU1 that had lost pCU1 and showed that these derivatives were genetically immune. He also hybridized restriction enzyme-digested total DNA of these derivatives with radio-labelled pCU1 DNA ("Southern"hybridizations) and this did not reveal DNA of the plasmid in these spontaneously cured strains. These observations were confirmed in the present study.

To serve as a positive control in these experiments and as shown in Materials and Methods, and Figure 4, a 400 bp fragment from pUC18Sfi (Herrero *et al.*, 1990) was inserted into the *Klebsiella* genome by first cloning it into the mini-Tn10 transposon in the suicidal vector pLOF/Km (Herrero *et al.*, 1990) and then inserting the transposon containing the 400 bp fragment into the genome of *Klebsiella*. The construct, denoted pMRK400 was transformed into the *E. coli* strain S17-1 λ pir which was used for transposon delivery (see Materials and Methods). pMRK400 was mated with UN2979, selecting for kanamycin resistant colonies. Total chromosomal

DNA was isolated from immune (M8; UNC109) and non-immune (M5a1; UN2979) *Klebsiella* strains and from one of the *Klebsiella* carrying the fragment from the transposition event involving pMRK400. The DNA was digested with *Hind*III and prepared for Southern hybridization as described in Materials and Methods. The 400 bp fragment was radioactively labelled and used as a probe. The results of hybridization are shown in Figure 5A. The probe was removed from the nylon membrane by treatment with alkali, the membrane was neutralized and reused in a second hybridization with radioactively labelled pCU1 DNA that had been digested, with *Hpa*I to provide a population of labelled pCU1 DNA fragments. The genomic DNA of none of the immune derivatives hybridized with the pCU1 probe (Figure 5B). Hybridization experiments similar to these were done with several other immune *Klebsiella* strains derived independently by Gill (1985). These yielded similar results. Immune derivatives were also derived from UN2979 cured of pCU403, pCU403-1, pCU56-13 and the small pCU1 derivative pCU1139 (pCU1107 carrying Kan/*oriT*, see Figure 12) from which the *Pst*I-*Hind*III fragment (radiolabelled) of pCU1107 was used as the probe. No hybridization was detected (results not shown). It is concluded that within the limits of 400 bp no part of pCU1 DNA exists in the immune *Klebsiella*.

Region of pCU1 needed for the detection and isolation of immune *Klebsiella*

Previous studies in this laboratory have resulted in a restriction map of pCU1 and in the isolation and mapping of the position of a large number of transposon

Figure 5. Southern blot of genomic DNA extracted from Imm⁻ and Imm⁺ *Klebsiella* digested with *Hind*III and hybridized with ³²P labelled probes.

Panel A: Hybridization with a 400 bp ³²P labelled probe from pMRK400

Panel B: Hybridization with ³²P labelled *Hpa*I fragments from pCU1

Lane A. DNA sample isolated from a Tn10::400 bp transposition derivative of *Klebsiella* UN2979.

Lane B. DNA sample isolated from Imm⁻ *Klebsiella* UN2979.

Lane C, D. DNA sample isolated from Imm⁺ *Klebsiella* cured of pCU109 (UNC109) and *Klebsiella* M5a1 cured of pCU1 (M8) respectively.

Lane E. DNA from bacteriophage Lambda digested with *Hind*III used as molecular weight standard.

Lane F. *Hind*III fragment containing the entire transfer region from pCU109 (used as positive control for panel B)

Lane G. 400 bp fragment from pUC18Sfi (used as positive control for panel A)

Arrows at the right indicate the position of the λ *Hind*III fragments which were in lane E. The molecular weight of the fragments in λ *Hind*III standard are from top to bottom in kb: 23.1, 9.4, 6.5, 4.3, 2.3, 2.0.

A B C D E F G

A

A B C D E F G

B

Tn5 insertions in the plasmid. Figure 6 shows the position of two Tn5 insertion derivatives, pCU66 at coordinate 30.2 and pCU88 at coordinate 10.2 in the pCU1 map. The figure also shows deletion derivatives originating from these insertions: pCU403, a deletion derivative originating from pCU66 by the removal of the pCU1 fragment from coordinates 21.0 to 30.2 and pCU109 (from pCU88) with coordinates 27.7 to 10.2 on the pCU1 map cloned as a *Hind*III fragment into the *Hind*III site of the plasmid vector pACYC184 (Chang and Cohen, 1978; Thatte, 1984). Also shown in this figure is the derivative called pCU56, containing the *Bgl*II fragment from coordinates 5.7 to 11.2 of pCU1 cloned into the *Bam*HI site of pACYC184 (Thatte, 1984). pCU1 and each of the derivatives pCU403, pCU109 and pCU56 were transferred into *Klebsiella* by mating and selection for the plasmid on minimal media with the appropriate antibiotic.

Since pCU56 is not a conjugative self-transmissible plasmid, it was transferred by conjugative mobilization using the helper plasmid pCU801 which cannot itself be transferred in this manner into the recipient strain because it has its own *oriT* region deleted (Rotheim *et al.*, 1988). *Klebsiella* strains carrying each of these plasmids were then tested for their ability to display immunity in a mating with either C600 /pCU1 or C600 /pCU109. In the case of *Klebsiella* carrying pCU109 or pCU403, the strains were also cured of the plasmids and tested again for immunity. pCU56 was the only derivative that did not confer immunity. From the results of these tests, it was inferred that the region shared by pCU403 and pCU109

Figure 6. Derivatives of pCU1 that were used to localize the region needed for the immune phenotype. The arrowheads indicate the location of the Tn5 insertions in the derivative pCU66 at coordinate 30.2 and pCU88 at coordinate 10.2. pCU56 is a derivative containig the *Bgl*III fragment from coordinates 5.7 to 11.2, cloned in the *Bam*HI site of pACYC184. The open rectangles within the pCU1 circle indicate the *tra* complementation groups (lettered), the antibiotic-resistance genes (Sp, Sm, Ap). The dark regions are the known *kik* regions and the dotted region, the origin of transfer (*oriT*). The open rectangles immediately outside the pCU1 map are respectively, the deletion derivative of pCU66 called pCU403, and the clones in pACYC184 (pCU109 and pCU56). The outermost stippled arc is the region that is inferred to be needed to confer immunity.

but absent in pCU56 was relevant to the appearance of the immune derivatives. This region is shown as a shaded area in Figure 6. Most of this shaded area is involved in conjugation (the *tra* region). In order to determine if any of the known *tra* genes could be involved in the immune phenotype, several Tn5 insertion derivatives of pCU109 referred to as pVT derivatives (Thatte *et al.*, 1985a) and located in different *tra* genetic complementation groups were used. In addition, a derivative called pCU812 constructed by Rotheim *et al* (1988) was also used. These Tra⁻ insertion derivatives of pCU109 and their respective location are shown in Figure 7. To transfer these Tra⁻ derivatives into *Klebsiella* by conjugative mobilization, the DNA of each plasmid insertion derivative was first transformed into *E. coli* HB101 carrying the helper plasmid pCU801. HB101 carrying both plasmids was then mated into *Klebsiella*. Transconjugants were selected on minimal agar containing kanamycin. The frequency of transfer for all the derivatives was low (approximately 10^{-7}) and similar to the frequency obtained with the self-transmissible plasmid pCU109. Transconjugants from each mating were purified twice on selective media and also in liquid minimal media containing kanamycin. The plasmid DNA was isolated from each derivative by the CsCl procedure described in Materials and Methods. Using appropriate restriction enzymes, the restriction pattern of each DNA isolated from the *Klebsiella* was compared to the same DNA isolated from the *E. coli* host. The *Klebsiella* derivatives were also tested for their immunity phenotype. The results shown in Table 4 indicated that all of the Tn5 derivatives

Figure 7. Transfer region of pCU1 present in pCU109 and the position of the transposon Tn5 insertions in the different Tra⁺ pVT derivatives. The rectangle represents the transfer region of pCU1 showing the coordinates (kb) from 30.0 to 11.0. The boxes represent the putative transcriptional units; the letters inside the box are the *tra* transcriptional units designated by Thatte *et al* (1985a). Arrows indicate the position of the Tn5 insertions in the Tra⁺ pVT derivatives of pCU109 and pCU812. *kikA* and *kikB* are the two regions the function of which have been implicated for the Kik⁺ phenotype. *oriT* is the origin of transfer. In the bottom figure, the dashed line is the cloning vector pACYC184 and IS indicates the small portion of IS50 that remains after the cloning.

Table 4. The immune phenotype of *Klebsiella* transconjugants derived from matings involving various pCU109 Tn5 insertion derivatives*

Derivative *	Map location of insertion (pCU1 coordinates)	Complementation Group	Immunity phenotype
pVT80	32.8	B	+
pVT70	33.5	B	+
pVT84	33.9	B	-
pVT83	34.2	B	+
pVT71	36.4	C	+
pVT96	38.0	D	+
pVT82	0.7	E	+
pVT61	2.5	F	+
pVT213	3.1	G	+
pVT149	6.5	H	+
pCU812	Tn5 insertion in <i>traA</i>	A	+

The source of insertion derivatives is described by Thatte *et al* (1985a) except for pCU812 which was constructed by Rotheim *et al* (1988).

*pCU109 is a derivative containing a continuous region of plasmid pCU1 cloned into the *Hind*III site of the vector pACYC184 (see Figure 6).

except one, conferred immunity. The exception was pVT84 that maps to the *tra* complementation group *traB* (Figure 7). The restriction pattern of pVT84 DNA isolated from the *Klebsiella* transconjugant was found to be altered when it was compared to the plasmid DNA isolated from the *E. coli* host; as many as six out of the eight *HpaI* fragments were missing from the derivative (Figure 8). Thus, none of these insertion derivatives defined a region that could be inferred unambiguously to be needed for immunity to arise. However, the results with pVT84 provided evidence that inactivation of a gene or genes in this region was destabilizing the plasmid.

Despite the large number *tra::Tn5* mutants analyzed, Thatte (1984) was unable to find any that could be mapped between *kikA* and the complementation group *traA*, or between the complementation groups C-D and G-H (Figure 7). One way to determine whether the presence of the region between complementation groups C and H, is necessary for immunity to arise was to analyze the behaviour in *Klebsiella* of an appropriate deletion derivative of pCU403 (described in Figure 9)

Construction of a deletion derivative of pCU403 and of the helper plasmid pVT149-1

Plasmid pCU403 is a derivative with a deletion that removes most of *kikA* (Figure 6) and which confers immunity to *Klebsiella*. pCU403 DNA was digested with *KpnI* and the products were separated on an agarose gel. The highest molecular

Figure 8. Agarose gel electrophoresis of *HpaI* restricted plasmid DNA isolated from *E. coli* and Imm⁻ *Klebsiella* pVT84 transconjugants.

Lane A. pVT84 isolated from Imm⁻ *Klebsiella* with only two *HpaI* fragments

Lane B. pVT84 isolated from *E. coli* showing the expected *HpaI* fragments

Lane C. DNA of bacteriophage Lambda restricted with *HindIII* (MW standards)

weight fragment was recovered from the gel by electroelution as described in Materials and Methods. The plasmid DNA was self-ligated *in vitro* and the ligation mixture was then used to transform *E. coli* HB101. Transformants were selected on LA containing kanamycin. Screening of several transformants revealed the expected restriction pattern and molecular weight of a pCU403 derivative that has lost its two internal *KpnI* fragments. The new plasmid is called pCU403-1 (Figure 9A)

pCU403-1 is transfer deficient and cannot be used for mobilization experiments using the helper plasmid pCU801 because both have the same replicon and are incompatible plasmids. In order to mobilize pCU403-1 into *Klebsiella*, it needed to be complemented by a compatible plasmid. The compatible helper was constructed by removing the *oriT* containing region from the plasmid pVT149 (Figure 9B). The Tn5 insertion that gave rise to pVT149 was located at coordinate 6.5 (in the pCU1 map) close to *oriT* (Figure 9B). *XhoI* restriction sites are located at the ends of the IS50 in Tn5 and also in the IS50 close to the replicon of the plasmid. Treatment of pVT149 with *XhoI*, removed the fragment of DNA containing part of *traH*, *oriT* and the *kikB* region (Figure 9B). The fragments were separated on an agarose gel and the highest molecular weight fragment was isolated from the gel by electroelution, self ligated and transformed into *E. coli* HB101 harbouring pCU403-1.

The constructed helper pVT149-1 (Figure 9B) was able to complement the *tra* functions missing in pCU403-1, allowing its transfer to either *E. coli* or to

Figure 9. Construction of pCU403-1, a *KpnI* deletion derivative of pCU403 and the helper plasmid pVT149-1. pCU403 and its derivative pCU403-1 have the replicon of pCU1 while pVT149 has the replicon of the compatible pACYC184.

A. pCU403 was constructed by deleting the pCU1 region from coordinates 21 to 30.2. The latter coordinate is within an IS50 region of a Tn5 insertion derivative called pCU66 that inactivated *kikA*. Thus pCU403 has only a small part of *kikA* (shown in this figure as '*kikA*). The region present in pCU403 is indicated by the partial circle immediately outside that of pCU1. pCU403-1 is the *KpnI* deletion derivative of pCU403, indicated by the outermost circle. Deleted regions are indicated by the gap. Other symbols are in Figure 6.

B. pVT149 (dark circle) is a Tn5 insertion derivative of pCU109. It has the entire *tra* region, the *kikA* and *kikB* regions and confers immunity to *Klebsiella*. The Tn5 insertion shown at coordinate 6.5 contains the IS50 (shaded rectangles) sequences containing the *XhoI* sites. The region between the *XhoI*₁ and the *XhoI*₃ sites close to the replicon which includes *oriT* was deleted to give rise to pVT149-1 (outer partial circle with deletion shown as a gap).

Klebsiella. The frequency of conjugative mobilization of pCU403-1 from *E. coli* to *E. coli* was similar to that of pCU403 (10^{-1}) and the frequency of conjugative mobilization from *E. coli* to *Klebsiella* when helped by pVT149-1 was similar to the frequency of conjugative transfer of the plasmid pCU403 (approximately 7×10^{-6}). Transconjugants were selected on glucose minimal media containing kanamycin and after three rounds of purification on selective media, the plasmid DNA was isolated from *E. coli* and *Klebsiella* transconjugants. Samples of DNA were restricted with *Bgl*III and the restriction pattern was analyzed and compared. The *Kpn*I deletion derivatives from pCU403-1 showed no structural change in *Klebsiella* and when the strain was cured of the plasmid all of the cured derivatives tested (10) were immune. These results indicated that in order for immune *Klebsiella* to arise from the population, the plasmid region between coordinates 30.2 (localization of the Tn5 insertion that gave rise to pCU403) to 34 (*Kpn*I site in pCU1) is relevant. This assumption was confirmed by the construction of pCU56-13.

A 6.9 kb fragment *Bam*HI-*Kpn*I from pCU403-1 containing the coordinates 30.2 to 34 from pCU1 and part of the Tn5 element was retrieved from pCU403-1 (see Figure 10) and cloned on the *Bam*HI-*Kpn*I sites of plasmid pUC128, to create pAAG13. Plasmid DNA isolated from this construct failed to transform competent *Klebsiella* cells under conditions when the DNA from the vector pUC128 (used as a control) gave 10^4 transformants/ μ g of DNA. The construction of a conjugatively mobilizable derivative of pAAG13 was accomplished by cloning a 5.9 kb fragment

Figure 10. Construction of pCU56-13, a conjugatively mobilizable derivative of pAAG13. pCU403-1 is a deletion derivative of pCU1. Regions present in it were shown in Figure 9. The *Bam*HI-*Kpn*I fragment including DNA from coordinates 30.2 to 34.0 of the pCU1 map was first cloned in pUC128 to give rise to pAAG13. The pUC128 portion in this derivative is shown as a thin line. In pAAG13, the open rectangle is part of the *tra* region (TraA and part of TraB, coordinates 30.2 to 34.0). The dark region is part of *kikA* and the shaded rectangle, IS50 from Tn5. pCU56 is a pACYC184 clone and the pCU1 region present in it is shown in Figure 5. It has a unique *Sph*I site into which the fragment retrieved from pAAG13 was cloned to give pCU56-13. The thin line in pCU56-13 is pCU56 and includes *oriT* and *kikB* of pCU1.

retrieved from pAAG13 (see Figure 10). pAAG13 was digested with *SphI*. In this plasmid one *SphI* site is located in IS50 (nucleotide 2121) and the other site, beside the *KpnI* site in the multicloning site of pUC128. The appropriate fragment was recovered from the gel and ligated into the *SphI* site of the plasmid derivative pCU56. Selection of transformants was on LA containing chloramphenicol. The resulting plasmid DNA from one of the transformants harbouring the insert, was called pCU56-13 and was used to transform the helper strain HB101 containing pCU801. The new construct was conjugatively mobilized into *Klebsiella* and transconjugants were recovered on glucose minimal plates containing chloramphenicol (100µg/ml). The frequency of transfer was low (10^{-7}) even though the frequency of transfer of pCU56 under the same conditions was 10^{-1} . Several transconjugants were tested independently for immunity with C600 (pCU1) as the donor. Two types of transconjugants were obtained one that was immune (Imm⁺) and one that was not (Imm⁻). When plasmid DNA was isolated from the *Klebsiella* transconjugants and their restriction pattern was analyzed, the Imm⁺ transconjugants showed a deleted pattern when it was compared to the plasmid DNA isolated from the Imm⁺ transconjugants or with the plasmid DNA isolated from *E. coli* transconjugants used as control. The ability of pCU56-13 to give rise to immune *Klebsiella*, was an indication that the relevant region might be located to the left of the first *KpnI* site, (reading clockwise) in pCU403-1 excluding the region between coordinate 34.0 and 10.2 (Figure 7)

All three insertion derivatives pVT70, pVT80 (in *traB*) and pCU812 (in *traA*) gave rise to immune *Klebsiella*. This suggested that the region relevant to immunity was between the site of insertion of pCU812 and *kikA*. A construct containing this region was available in our laboratory; pCU1106 was constructed by L. Ailles (unpublished results) by digesting the plasmid DNA from pCU171 (a Tn5 insertion in *traA* of pCU1) with *Hind*III and *Xho*I and cloning the pCU1 DNA fragment from coordinates 27.7 (*Hind*III site) to 32.4 (*Xho*I site within the Tn5 in pCU171) into the plasmid vector pACYC184. The site of insertion of pCU812 in *traA* (Figure 7) is identical to that of pCU171 (Figure 12), the former having been derived from the latter (Rotheim *et al.*, 1988).

This plasmid was further reduced in size by digesting pCU1106 with *Sal*I. The digestion removed the pCU1 DNA from coordinates 27.9 to 28.8 producing the derivative pCU1107. Plasmid DNA isolated from a strain containing pCU1107 was unable to transform *Klebsiella*, although the vector pACYC184 did so efficiently.

I then used similar derivatives containing in addition the origin of conjugal transfer (*oriT*) of pCU1 (called pCU1108) or *oriT* of RK2 (called pCU1139 and pCU1140, derivatives with the *oriT*_{RK2} cassette located in different orientation (Hengen, 1991). These plasmids were used to transform the helper strain HB101/pCU801 for pCU1108 and S17-1 for pCU1139 and pCU1140.

The plasmid derivative pCU1108 was not mobilized by the helper plasmid pCU801; presumably, during its *in vitro* construction, part of the *mob* region needed

Table 5. Frequency of mobilization of plasmid pCU1139 and pCU1140 to *E. coli* and *Klebsiella oxytoca* recipients.

Donor plasmid in <i>E. coli</i> S17-1	Marker selected in conjugation	Frequency of Transfer to*	
		<i>E. coli</i>	<i>K. oxytoca</i>
pCU1139	Km	1.0×10^{-1}	2×10^{-6}
pCU1140	Km	1.2×10^{-1}	2×10^{-6}

* Matings were performed at donor to recipient ratios of 1:10 on membrane filters and incubated on a prewarmed plate at 37°C for one hour. Cells were resuspended in saline and dilutions were plated on LA with rifampicin (100µg/ml) and Kanamycin (30µg/ml). The numbers are the average of the frequency of transfer in two experiments. The *E. coli* used as recipient was HB101Rif^r and the *Klebsiella* was UN2979Rif^r.

for the initiation of its transfer from *oriT* had been deleted. S17-1 donors containing pCU1139 or pCU1140 were mated with *E. coli* recipient (results shown in Table 5) and the transfer frequency was similar to the frequency obtained in the transfer of plasmid RP4 when it is in an autonomous state in a similar donor (data not shown). When the two derivatives were transferred to *Klebsiella* recipients under the same conditions, the frequency of transfer was 10^5 fold less. The transconjugants were purified and analyzed for the immune phenotype. Both derivatives gave Imm⁺ and Imm⁻ transconjugants, the latter group comprising 10 to 12% of the tested transconjugants (total, 100 each). It could also be concluded that Imm⁺ and Imm⁻ transconjugants were obtained regardless of the orientation of the 4.5 kb Kan/*oriT* fragment (in pCU1139 and pCU1140 respectively).

A relevant observation from the experiments, the results of which are presented in Table 5, was that plasmid DNA reisolated and examined from the Imm⁻ transconjugants, showed evidence of DNA insertion or rearrangements, while plasmid DNA from Imm⁺ transconjugants did not. The plasmid DNA of Imm⁻ transconjugants that had undergone major rearrangements was not analyzed further. Some Imm⁻ transconjugants had plasmid DNA that appeared to have simple insertions. Two of them called pCU11403 and pCU11404 had an insertion of approximately 1 kb. Figure 11 shows the restriction pattern of one of these derivatives (pCU11403). In a subsequent section, the region of the insertions has been identified by nucleotide sequencing.

Figure 11. Agarose gel electrophoresis of *Hind*III + *Ssp*I restricted plasmid DNA isolated from Imm⁺ and Imm⁻ *Klebsiella* transconjugants from pCU1140.

Lane A. DNA of Bacteriophage Lambda restricted with *Hind*III (MW standards)

Lane B. Plasmid DNA isolated from Imm⁺ transconjugant (pCU1140A)

Lane C. Plasmid DNA isolated from Imm⁻ transconjugant (pCU11403), the fifth fragment had an increment of size now located as a new fragment of 2.2 kb (indicated by the arrow)

Lane D. 1 kb DNA MW standards.

Plasmid DNA isolated from some Imm⁺ and Imm⁻ *Klebsiella* transconjugants was next used to transform *E. coli* S17-1 in order to subsequently determine if they had undergone any change in their ability to be conjugatively mobilized. The results presented in Table 6, indicate that the Imm⁻ derivative pCU1140-A shows the same frequency of mobilization as pCU1140 and the analysis of 50 independent and purified transconjugants from this experiment indicated that 6/50 were Imm⁻. The plasmid DNA from the Imm⁻ insertion derivatives pCU11402 and pCU11403 gave frequencies of transfer comparable to the values obtained with *E. coli* as a recipient (i.e. They had gained the ability to yield a high frequency of transconjugants). Plasmid DNA of pCU11402 and pCU11403 were also able to transform competent *Klebsiella* cells with a high frequency similar to the vector pUC128.

The Imm⁻ plasmids showing both a high frequency of conjugative mobilization and transformation were considered to be candidates with a mutation in a region that potentially reduced the viability of *Klebsiella* or the survival of the plasmid. In order to localize this region, pCU1107 (see Figure 12) was linearized with *Hind*III and digested with *Bal*31 as described in Materials and Methods. The DNA was end-filled with Klenow enzyme and the four dNTPs, *Hind*III linkers were ligated at the ends and the excess was removed by digestion with the same enzyme. The DNA was isolated and circularized by T4 DNA ligase. The ligation mixture was used to transform *E. coli* DH5 α cells and transformants selected for chloramphenicol-resistance. To further reduce the region in the IS element and to

Table 6. Frequency of mobilization of pCU1140 derivatives isolated from Imm⁺ and Imm⁻ *Klebsiella* transconjugants.

Donor plasmid in <i>E. coli</i> S17-1	Immune phenotype of <i>Klebsiella</i> from which the plasmids were isolated and transformed in S17-1	Frequency of transfer to <i>Klebsiella oxytoca</i>
pCU1140-A	Imm ⁺	6.0×10^{-6}
pCU11402	Imm ⁻	0.6×10^{-1}
pCU11403	Imm ⁻	1.0×10^{-1}

retrieve the fragment, the *Bgl*I site located at nucleotide 165 of IS50 was replaced by *Pst*I, by adding *Pst*I linkers. The *Bal*31-deleted pCU1107 plasmid DNA, was digested with *Hind*III and *Pst*I and the resulting fragment was cloned into the *Hind*III-*Pst*I sites of pUC128, to generate the plasmid pAAG8. This construct was isolated in *E. coli* selecting for ampicillin resistant transformants. The region of pCU1 present in pCU1106, pCU1107 and pAAG8 is shown in Figure 12.

The plasmid DNA isolated from pAAG8 was unable to transform *Klebsiella*. It was then mobilized into *Klebsiella* by first cloning the *oriT*_{RK2} cassette into the *Hind*III site as described previously, and the ligation mixture being transformed into the helper strain S17-1. The new construct denoted pAAG845 was introduced by conjugative mobilization into *Klebsiella* (UN2979) and the transconjugants selected on LA containing rifampicin and kanamycin. The frequency of transfer was similar to that obtained with pCU1140. Imm⁺ and Imm⁻ transconjugants were again obtained in a similar ratio. One of the Imm⁻ transconjugant that was analyzed showed a restriction pattern different from the plasmid DNA isolated from an Imm⁺ transconjugant, as in the case of pCU11403 (Figure 11). The alteration consisted of an insertion of an extra fragment of DNA of approximately 1 kb (result not shown).

Figure 12. Partial map of pCU1 showing the deletion derivatives pCU1106, pCU1107 and pAAG8. The numbers indicate the kb coordinates of pCU1; only four of the eight complementation groups are shown as boxes with the letters. The solid line represents the pCU1 region present in the derivatives. The triangle represents the Tn5 insertion at position 32.4 in pCU171. The IS50 region in pAAG8 has been reduced to 167 bp by adding a *Pst*I linker at the *Bgl*II site.

Nucleotide sequence analysis of the 2.34 kb fragment of pCU1 present in pAAG8

The nucleotide sequence of pCU1 present in pAAG8 a relatively small plasmid that could give rise to Imm⁺ (approximately 88%) and Imm⁻ (approximately 12%) *Klebsiella* was determined on both strands. The sequencing was preformed by the dideoxy-chain termination method, using double stranded DNA template and the modification suggested by Disotell *et al* (1992), as described in Materials and Methods. The M13-reverse primer was used to map the Tn5 insertion in the *traA* region and a primer complementary to the end of the IS50 was used to map the position of the Tn5 insertion present in *kikA* of pCU403 using the plasmid pAAG13. After the first round of sequencing, synthetic oligonucleotide primers were used to sequence from both strands the DNA present in pAAG8. Problems due to marked tendencies of some regions to form secondary structures that were difficult to read were overcome by using nucleotide analogs (see Materials and Methods). The sequencing strategy used is outlined in Figure 13.

In order to provide the complete nucleotide sequence present in pAAG8 (Figure 14), the nucleotide sequence from position 2160 (where the *kikA* insertion was mapped in pCU403) to 2340 was taken from the *kikA* sequence published by Hengen *et al* (1992a). Coordinates for the location of recognition sites for restriction endonucleases with one or two sites in the sequence are described in Table 7.

Description of the pCU1 sequences present in plasmid pAAG8

The pCU1 sequences present in pAAG8 were examined in both directions for open reading frames (ORFs) of at least 60 coding triplets starting with either ATG or GTG, preceded by a reasonable Shine-Dalgarno sequence (Shine and Dalgarno, 1974), and terminated with a stop codon. Besides the three ORFs previously identified by Hengen (1991) in the *kikA* region, twelve other potential ORFs (Figure 15) were identified by computer analysis. Table 8 lists coordinates for their translational start and stop codons and also the calculated molecular weight of these predicted polypeptides. Seven of these ORFs (ORF115, ORF62, ORF60, ORF63, ORF123, ORF101 and ORF125) read in the same direction as *kikA* and begin with ATG. An initiation codon GTG is present in the same frame as ORF115 at position 84. Another GTG codon with a Shine-Dalgarno sequence is located at position 481 for ORF62. The analysis of ORF101 indicates a start site at position 1359 with GTG and at another position 1362 with an ATG site; in both cases, the sequence GAAGGAAGGAA located between nucleotide 1347 and 1355 with homology to the Shine-Dalgarno sequence could be used for the two initiation codons. There are two stop codons (UGA, UAA) in tandem at the end of ORF63 but with an overlap of the first stop codon and a start codon at the junction of ORF63 and ORF123, an observation that raises the possibility that the synthesis of these polypeptides could be coupled (Gold, 1988). The ORF101 protein which is in frame with ORF123 can be made from an independent transcript as there are appropriate -10 and -35

Figure 13. Illustration of the strategy used to sequence the pCU1 DNA present in pAAG8. The central bar represents the region of pCU1 present in pAAG8. Sequencing was progressive and on double-stranded plasmid DNA using synthesized primers. The horizontal arrows and their length indicate the direction and the extent of unambiguously readable sequence information from the autoradiograms. The triangles indicate the location of the Tn5 insertion in *traA* and *kikA* from which the sequencing was initiated and the numbers, kilobases of sequence from the position of the Tn5 insert (pCU171) in *traA*.

Table 7. Restriction endonucleases with one or two sites in the pCU1 region present in pAAG8

Enzyme insertion	# sites	Location, nucleotide from the Tn5 site in pCU1107
<i>AhaI</i>	1	630
<i>Avall</i>	2	1,081 1,588
<i>BanI</i>	2	131
	1	629
<i>BdeI</i>	1	633
<i>BsmI</i>	1	2,260
<i>BspI286</i>	1	88
<i>BspMI</i>	2	829
	1	896
<i>ClalI</i>	2	1,081 1,588
<i>FspI</i>	1	1,152
<i>HaeI</i>	1	1,216
<i>HincII</i>	2	349 917
<i>HpaI</i>	1	349
<i>MaeI</i>	1	2,234
<i>NarI</i>	1	630
<i>PvuII</i>	2	379 2,051
<i>SspI</i>	2	1,067 1,956
<i>XhoII</i>	2	475 2,091

Figure 14. Nucleotide sequence of pAAG8. The numbers indicate nucleotide positions. Putative promoter sequences (-35 and -10) and putative Shine-Dalgarno sequences (SD) are underlined. Right arrows indicate ORFs from *traA* towards *kikA* upper strand, left arrows indicate ORFs in the lower strand. Initiation codons are represented in bold. Stop codons for the ORFs in the upper strand are represented by an asterisk (*) and the ones in the lower strand by a downward arrow (↓). Dots above the nucleotide sequence indicate *dam* methylation sites. The bars (DR1 and DR2) above the sequence indicate the direct repeats.

10 20 30 40 50 ... 60
GAATAAAGCT GGCTGGGATT ACGTGAAGCA TCCACATAAA GCAACTAACC ATGATCGCAA
SD → ORF115
CTTATTTCTGA CCGACCCTAA TGCACCTTCGT AGGTGTATTT CGTTGATTGG TACTAGCGTT

70 80 90 100 110 120
TAGCACAACC AATTGGAATC CATGTGCTCA ACCACGTCTG GATTGATGTA GCGGTTGATT
ATCGTGTTGG TTAACCTTAG GTACACGAGT TGGTGCAGAC CTAACCTACAT CGCCAACTAA

130 140 150 160 170
CACCAGTATC GGTGCCAGCG GCCAGCGCAA TTTGCGGCAG GGCATGGAC AAAACGCCCCA
GTGGTCATAG CCACGGTCGC CGGTCGCGTT AAACGCCGTC CCGTTACCTG TTTTGCGGGT

DR2
190 200 210 220 230 240
TAACTACCGC AGGGCCATAC TTCTTAAACA ACGTGGTCAT ACTTCTCCT TACTCACTAT
ATTGATGGCG TCCCGGTATG AAGAATTTGT TGCACCAGTA TGAAAGAGGA ATGAGTGATA

250 260 270 280 290 300
CTTCTGTTGA TTGCAGATTT AACCGGCATT CTTCAACGCC ACACTTATCT TTCAAAACT
GAAGACAACCT AACGTCTAAA TTGGCCGTAA GAAGTTGCGG TGTGAATAGA AAGTTTTTGA

310 320 330 340 350 360
CTGCCAGTAG CCTTAAATCA GCCCAGGTTT GTAGCTCATG CTCTTTGTTA ACCCGAATGG
SD
GACGGTCATC GGAATTTAGT CGGGTCCAAG CATCGAGTAC GAGAAACAAT TGGGCTTACC

370 380 390 400 410 420
AACATGCGAT CTGGCCCAGC TGTTTCATCGT CTATCTGAAA AACGGGATAC CACTTCTTTA
→ ORF62
TTGTACGCTA GACCGGGTCG ACAAGTAGCA GATAGACTTT TTGCCCTATG GTGAAGAAAT

430 440 450 460 470 480
ATGAAACCCG TTCTATCGTT ACCTCCTTCA CTTTTCCTGC TTCAAATAAC TCCAGGATCT
TACTTTGGGC AAGATAGCAA TGGAGGAAAGT GAAAAGGACG AAGTTTATTG AGGTCCTAGA
ORF73 ← SD -10

490 500 510 520 530 540
GTGATTGCAA TACAAGATAC AAACGTTCCTA TTCATAACCT CATTCCCCCT TCGCTGTTTC
CACTAACGTT ATGTTCTATG TTTGCAAGGT AAGTATTGGA GTAAGGGGGA AGCGACAAAG
-35 ORF93 ← SD

550 560 570 580 590 600
CTGTCTGTT AAAAAGGCAT CCGTATTATT TCGGCTGAAG GCATCGCCAT CACCAGAACC
↓ *
GACAGACCAA TTTTCCGTA GGCATAATAA AGCCGACTTC CGTAGCGGTA GTGGTCTTGG
-10 -35

610 620 630 640 650 660
 AAAAACATCT TGTTACCCGT CATACTGCGG CGCCGTACTT TTTGCCCTGCT GAGGCTCATT
TTTTTGTAGA ACAAGTGGCA GTATGACGCC GCGGCATGAA AAACGGACGA CTCCGAGTAA
 670 680 690 700 710 720
 CGCGGTGCTG TCCTCACTGG TCTGGCCGTC AGTAGCAGCG TAGGGATTTT CAAATCAGTT
 GCGCCACGAC AGGAGTGACC AGACCGGCAG TCATCGTCGC ATCCCTAAAA GTTTAGTCAA
 730 740 750 760 770 780
 GATTGACGCG CCACGTTGAT AACTTTCGTG ACATACCCGT TAGAAATCCC GTTTGTGAGT
 CTAAGTGC GC GGTGCAACTA TTGAAAGCAC TGTATGGGCA ATCTTTAGGG CAAACACTCA
 790 800 810 820 830 840
 GAGCCGGTGT TGTAGCAGGA AAGCGCGTGT CTCAGCGCAA CCTGCCCGGC TGGGTAGGAT
 CTCGGCCACA ACATCGTCCT TTCGCGCACA GAGTCGCGTT GGACGGGGCCG ACCCATCCTA
 850 860 870 880 890 900
 TTCAGGGCGC TATCATAACA GGCTTTAAGA ATGGTCTGGC TCGCCCGCAG GTTGATGCAG
 AAGTCCCGCG ATAGTATTGT CCGAAAATTCT TACCAGACCG AGCGGGCGTC CAACTACGTC
 910 920 930 940 950 960
 GGTTTGAAAA TATCGTCAAC CGAAAGACCC AGGCCCACTA AATTATTGTA GTTAATTGTT
 CCAAACTTTT ATAGCAGTTG GCTTTCTGGG TCCGGGTGAT TTAATAAACT CAATTAAACA
 970 980 990 1000 1010 1020
 GCAAGGCCCA TATCAAACT TTTATTATCC TTCAGCAGAA CTTTCGCAAC GCTGACGGCC
 CGTTCCGGGT ATAGTTTTGA AAATAATAGG AAGTCGTCTT GAAAGCGTTG CGACTGCCGG
 DR1 DR2
 1030 1040 1050 1060 1070 1080
 TCAGCTTCAG TACGCGGTTG CTGTTTAAAC TGAATACTAC CGTTAATATT GATCCTGTAC
 AGTCGAAGTC ATGCGCCAAC GACAAAATTG ACTTATGATG GCAATTATAA CTAGGACATG
 1090 1100 1110 1120 1130 1140
 GGTCCATTTG AGGACTCATG GCCGACGATG TACGCCATCG TTAAGGGTGA AACATCAGGC
 CCAGGTAAAC TCCTGAGTAC CGGCTGCTAC ATGCGGTAGC AATTCCCACT TTGTAGTCCG
 1150 1160 1170 1180 1190 1200
 GCACATCTCT GCGCAAGCCT GGCAACTTCA TCGGAGGCAG GCGCAGCACT GGCACGGCCA
 CGTGTAAGAGA CGCGTTCCGA CCGTTGAAGT AGCCTCCGTC CGCGTCGTGA CCGTGCCGGT

1210 1220 1230 1240 1250 1260
GCACAAGCAA GGCAGGCCAG AGCGAGAACC AGGAGTTTTG GATGTTTACT CATTTATGTT
CGTGTTTCGTT CCGTCCGGTC TCGCTCTTGG TCCTCAAAAC CTACAAATGA GTAAATACAA

ORF234 ←

1270 1280 1290 1300 13101320
TCACCGCGTA ATTGTAATTT CGACGACTTT TGTTTATGAT AAGGGCTAAT GAGATCGTGT
-35 -10* *

→ ORF123

AGTGGCGCAT TAACATTAAA GCTGCTGAAA ACAAATACTA TTCCCGATTA CTCTAGCACA
SD -10

1330 1340 1350 1360 1370 1380
CAAGAAGAAA CGAAAAGAAA ACCGCTGAAG GAAGGAAGGT GATGCTGGTT CCCCTGAAAT
SD → → ORF101
GTCTTTCTTT GCTTTTCTTT TGGCGACTTC CTTCCCTTCCA CTACGACCAA GGGGACTTTA
-35

1390 1400 1410 1420 1430 1440
CAGAAAAACG ACCAAAAGGC GAACCCGTGT ATCGTGACCC GGATAACCCT TTTAATACGT
GTCTTTTTGC TGGTTTTCCG CTTGGGCACA TAGCACTGGG CCTATTGGGA AAATTATGCA

1450 1460 1470 1480 1490 1500
GGAAGCTGAT AGGGAAGCGC CCGGCCTGGC TAACTGCAAA ATTGGACGCT GGCATTAGCC
CCTGACCATA TCCCTTCGCG GGCCGGACCG ATTGACGTTT TAACCTGCGA CCGTAATCGG

1510 1520 1530 1540 1550 1560
TGGAAGCCAT GAAGATGCAG GGCCTAGCCA ACCCCAGAGA ACATAGACCA GCAAAATACC
ACCTTCGGTA CTTCTACGTC CCGCATCGGT TGGGGTCTCT TGTATCTGGT CGTTTTATGG

1570 1580 1590 1600 1610 1620
GCGACCCCAG GAACGCAGAA AATACCTGGT CCGGGACTGG CCGCCGACCC ACATGGCTCA
CGCTGGGGTC CTTGCGTCTT TTATGGACCA GGCCCTGACC GGCGGCTGGG TGTACCGAGT

1630 1640 16501660 1670 1680
AAGAGCTGCT TGATAGTGGT TTATCACTTG ATGATCTGAA GATATAACCG GAGGGTATAA
TTCTCGACGA ACTATCACCA AATAGTGAAC TACTAGACTT CTATATTGGC CTCCCATATT
SD

1690 1700 1710 1720 1730 1740
AAATGGGCGA ACTTATTGAT TTTGCGGAAA GGCAAAAAAG CAGAAGGAAG AAAAAAGCAT
→ ORF125
TTTACCCGCT TGAATAACTA AAACGCCTTT CCGTTTTTTC GTCTTCCTTC TTTTTTCGTA

1750 1760 1770 1780 1790 1800
CTTCCATTCC GCCCATTTTT AGAAAATTTT GTGTCCATGC GATCAGGTTA CTCGCCAGCA

GAAGGTAAGG CCGGTAAAAA TCTTTTAAAG CACAGGTACG CTAGTCCAAT GAGCGGTCGT
 ORF165 ORF184

1810 1820 1830 1840 1850 1860
 TCATCAAATC CGGCTCTTAT TCAGTTGCTT ATATCGTTAA AAAAATTACA GGAAAGTTAA
 AGTAGTTTAG GCGGAGAATA AGTCAACGAA TATAGCAATT TTTTAAATGT CCTTTC AATT
 SD -10

1870 1880 1890 1900 1910 1920
 TCAAGTTTAA CACAATTTTA ACGATTTTCA TTTTGTGTGT CGAATATATT GCAGGCCGATA
 AGTTCAAAT GTGTTAAAAT TGCTAAAAGT AAAAACAACA GCTTATATAA CGTCCGCTAT
 -35

1930 1940 1950 1960 1970 1980
 TAGGTTACAA ATCCATTTAT AACGCAGCGT TATTATTAAT ATTACTTACC TTTATCAACA
 -35 -10
 ATCCAATGTT TAGGTAAATA TTGCGTCGCA ATAATAATTA TAATGAATGG AAATAGTTGT

1990 2000 2010 2020 2030 2040
 TTCTGGCGAG TCATATCTG AACAACTGT TAAGGACAAA AAATGAAGAA ACTCTTAATA
 SD → ORF104
 AAGACCGCTC ACATATAGAC TTGTTTGACA ATTCCTGTTT TTTACTTCTT TGAGAATTAT

2050 2060 2070 2080 2090 2100
 CCTCTGATAG CAGCTGGTAG TCTGCTTTAT CTTCCTGCCA GCCATGCTGA AGATCCCTGC
 * ↓ → ORF70
 GGAGACTATC GTCGACCATC AGACGAAATA GAAGGACGGT CGGTACGACT TCTAGGGACG

2110 2120 2130 2140 2150 2160
 AAAGTTATTA TGTGCATGGC GGGCAAGCTC ACCGGCGATA GCGGCGGAAG CGAGTGTAAC
 TTTCAATAAT ACACGTACCG CCCGTTGAG TGGCCGCTAT CGCCGCCTTC GCTCACATTG

2170 2180 2190 2200 2210 2220
 AGTGCTGAAG CTGCTTTCTT CAATATCGTT AAAAAGAACA AGCACGGCTT TTTACCCAAC
 TCACGACTTC GACGAAAGAA GTTATAGCAA TTTTCTTGT TCGTGCCGAA AAATGGCTTC

2230 2240 2250 2260 2270 2280
 CACACGAAGG ATGCTAGGAA GGCTTTTCTT AATGAATGCC CGGATAATGG CGAAGGTGGA
 GTGTGCTTCC TACGATCCTT CCGAAAAGAA TTACTTACGG GCCTATTACC GCTTCCACCT

2290 2300 2310 2320 2330 2340
 AATAACCAAGT CGATGATAAG CCAGATCATA AGTAAATACG GGAAAGTTTC CTTATAGGCC
 * *
 TCATTGGTCA GCTACTATTC GGTCTAGTAT TCATTTATGC CCTTTCAAGC GAATATCCGG
 ORF80 ←

Table 8. Characteristics of the predicted ORFs present in pAAG8

ORFs present in upper strand towards <i>kikA</i>	location	Molecular weight (Da)	# of amino acids
ORF115	51-398	12,326	115
ORF62	364-552	6,784	62
ORF60	871-1053	6,776	60
ORF63	1108-1299	6,711	63
ORF123	1296-1667	14,298	123
ORF101*	1362-1667	11,140	101
ORF125	1683-2060	16,510	125
ORF104	2023-2337	11,140	104
ORF70	2083-2296	7,740	70
ORFs present in lower strand towards <i>traA</i>			
ORF80	2309-2067	8,774	80
ORF184	1804-1254	20,196	184
ORF165	1747-1254	18,106	165
ORF234	1253-549	25,316	234
ORF93	511-230	10,957	93
ORF73*	441-230	8,594	73

* GTG initiation codon

Figure 15. Open reading frames detected in the pCU1 sequence present in the plasmid pAAG8. The numbered line is the sequenced region with the top bar showing the coordinates of the parental pCU1 map and the second line kilobases from the unique *Pst*I site in pAAG8. The relevant potential ORFs on both strands are shown with an arrow indicating the orientation of transcription. Branched arrows indicate a potential ORF in the same frame. The arrowhead between ORF63 and ORF123 indicates a potential start site (for ORF123) that is one base out of frame with the first potential stop codon for ORF63. The open box indicates the *kikA* region that was sequenced and analyzed by Hengen *et al* (1992a).

sequences as well as a good ribosome binding site for it (Figure 14 and Figure 20).

In the lower strand five ORFs were detected: ORF184, ORF165, ORF234, ORF93 and ORF73 reading in the direction towards *traA* (Figure 14, see also Figure 15). ORF184 and ORF165 are located in the same frame both with ATG start codons but with a Shine-Dalgarno sequence only nine bp apart from the start codon of ORF184. ORF93 and ORF73 are in the same frame, ORF93 initiated with ATG at position 511 and ORF73 with GTG at position 451, both with Shine-Dalgarno sequences located six and three bp respectively from the respective initiation codons.

Promoter-like sequences related to the consensus sequence for the σ^{70} promoter (Hawley and McClure, 1983) were found on both strands. A putative promoter sequence is located in the upper strand at position 1038-1069 close to ORF63 and another one at position 1272 to 1300 probably transcribing ORF101. In the lower strand four promoter-like sequences were located. At position 1834-1864 before ORF184, another promoter-like sequence is at position 1296 to 1323 with the -10 hexamer overlapping the -10 hexamer of the promoter located in the upper strand; this promoter could be the one allowing transcription of ORF234. At position 574 to 602 before ORF93 there is another sequence which could be acting as promoter even though there is not a perfect match with the σ^{70} promoter of *E. coli* and the last one is at position 464-490 before ORF73. The sequence also has several GATC sites which could be involved in methylation; they are located at position 53, 368, 478, 1071, 1313, 1653, and 1781. There is one pair of direct repeats of ten nucleotides

each (DR1) located at nucleotide 108-117 and 1029-1040, with the sequence GTAGCGGTTG; and another pair of direct repeats of nine nucleotides each (DR2) located at nucleotide 180-189 and 1054-1062, with the sequence ATACTACCG.

From a computer search of the peptide sequence databanks (searched with the programs Blast and Tfasta) of all the ORFs present in pAAG8, only ORF234 showed significant homology with the VirB1 protein precursor of the *Agrobacterium* plasmid pTiA6NC (Ward *et al.*, 1988). The region of similarity is shown in Figure 16. The two sequences had 63 identical residues and 37 partial residues, when two gaps are introduced in the alignment of the two sequences. The computer analysis of hydrophobic and hydrophilic amino acid residues using the algorithm developed by Kyte and Doolittle (1982) shown in Figure 17, revealed a hydrophobic domain within the first 20 amino acids of ORF234, suggesting a possible export signal sequence and its association with the inner membrane. The prediction of signal sequence cleavage sites based on the method of von Heijne (1986) revealed at the N-terminus, a putative signal peptide with a potential cleavage site located at amino acid position 26. These two features are also described for VirB1 (Ward *et al.*, 1988; Shirasu *et al.*, 1990). Contrary to VirB1 from *Agrobacterium* that has a extremely hydrophilic C-terminal region, ORF234 has a hydrophobic carboxyl terminus. The VirB proteins are suggested to act at the bacterial surface and to play an important role in directing T-DNA transfer from *Agrobacterium* to plant cells during conjugation (Shirasu *et al.*, 1990). The similarity of ORF234 with a protein

Figure 16. Alignment of ORF234 to the VirB1 protein of *Agrobacterium tumefaciens* plasmid pTiA6NC. Alignment of sequences with the introduction of two gaps. There are 63 identical matches (*) and 37 similar residues (|), similar to those reported by Ward *et al* (1988). Downward arrowhead indicates predicted signal peptidase cleavage site by the method of von Heijne (1986).

Vir Bl..1. M F K R S G S L S L A L M S S F C S S S L A T P L S S A E
 * * | | * * * * * * | | | * *
 ORF234..1. M S K H P K L L V L A L A C L A C A G R A S R A P A S D E

Vir Bl.. F D H V A R K C A P S V A T S T L A A I A K V E S R F D P
 | | * | | * * * * * * | * * * *
 ORF234.. V A R L A Q R C A P D V S P L T M A Y I V G H E S S N G P

Vir Bl.. L A I H D N T T G E - T L H W Q D H T Q A T Q V V R H R L
 * | * * | * * * * * * | * * *
 ORF234.. Y R I N I N G S I Q L K Q Q P R T E A E A V S V A K V L L

Vir Bl.. D A R H S L D V G L M Q I N S R N F S M L G L T P D G A L
 * * * | * * * * * * * * * *
 ORF234.. K D N K S F D M G L A Q I N S N N L V G L G L S V D D I F

Vir Bl.. K A C P S L S A A A N M L K S R Y A G G - E T I D E K Q I
 * | * | * * * | * * * * * * | *
 ORF234.. K P C I N L R A S Q T I L K A C Y D S A L K S Y P A G Q V

Vir Bl.. A L R R A I S A Y N T G N F I R G F A N G Y V R K V E T A
 * * * | * | * * * * * * * | * * * * *
 ORF234.. A L R H A L S C Y N T G S L T N G I S N G Y V T K V I N V

Vir Bl.. A Q .. 174
 * |
 ORF234.. A R .. 176

Figure 17. Hydropathy plot analysis of the putative ORF234 product.

Hydrophobic score was calculated using the Kyte-Doolittle scale and averaging over a window of eleven amino acid residues. Hydrophobic regions are represented by a positive index score.

constituting part of the transfer region like VirB1 implies a role for ORF234, in bacterial conjugation. Although it has not previously been considered to be part of the *tra* region of pCU1(Thatte *et al.*, 1985a). A similar sequence is also present in the IncN plasmid pKM101 (Winans, pers. commun). Although, as in the case of pCU1, the published *tra* region of this plasmid (Winans and Walker, 1985b) does not include this sequence.

Deletion derivatives of pAAG8

Using the information provided by the sequence, a series of deletion derivatives of pAAG8 was constructed as described briefly in Table 9 and the lower part of Figure 18. As most of these derivatives were unable to transform *Klebsiella*, the 4.5 kb Kan/*oriT*_{RK2} cassette was linked *in vitro* to each of them and the resulting plasmid (identified by the ending numeral 45) mobilized from the helper *E. coli* S17-1 to *Klebsiella*. In each case, the mobilization frequency was low (approximately 10^{-7}) and they gave rise to Imm⁺ (88-90%) and Imm⁺ *Klebsiella* (10-12%). Plasmid DNA reisolated from the Imm⁺ *Klebsiella* were found to be highly efficient in transformation reactions and to have mutations in their DNA (some of which have been mapped in a subsequent section). Plasmid DNA from Imm⁺ *Klebsiella* were as a rule unchanged in their restriction pattern (enzyme used, *Hind*III and *Ssp*I) and gave rise again to Imm⁺ and Imm⁺ *Klebsiella* in

Table 9. Derivation of progressive deletions from pAAG8 and pAAG5. Ability of the derivative to transform *Klebsiella*.

Plasmid	Description	Transformation in <i>Klebsiella</i>
pAAG8/ pAAG845*	2,500 bp of pCU1 from the Tn5 insertion in <i>traA</i> to 160bp after the <i>kikA</i> region	-
pAAG7/ pAAG745*	Bal31 derivative of pAAG8 from the <i>Hind</i> III site. The deletion ended at nucleotide 1,842	-
pAAG6/ pAAG645*	As above, the deletion ended at nucleotide 1,754.	-
pAAG5	cloning of the <i>Pst</i> I- <i>Hind</i> III fragment from pAAG6 into the <i>Pst</i> I- <i>Hind</i> III site of pUC128 vector.	-
pAAG4/ pAAG445*	Bal31 deletion from the <i>Hind</i> III site of pAAG5 Deletion end point at nucleotide 1,597	+
pAAG3/ pAAG345*	Same as above. The deletion ended at nucleotide 1,390	+
pAAG2/ pAAG245*	Same as above. The deletion ended at nucleotide 1,158	+

continued..

Table 9. continuation

pAAG10	<i>SspI</i> deletion derivative of pAAG5, from nucleotide 1754 to 1,067 (<i>SspI</i> site)	+
pAAG15	<i>NarI</i> deletion derivative of pAAG5, from nucleotide 1754 to 630 (<i>NarI</i> site)	+
pAAG15-1	Bal 31 deletion derivative of pAAG15 removing from nucleotide 630 to 601	+
pAAG1	Derivative isolated from pAAG5 by deletion from the first nucleotide (<i>PstI</i> site) to the <i>HincII</i> site at position 917.	-
pAAG1-184	The <i>BamHI-HindIII</i> fragment was retrieved from pAAG1 by <i>BamHI-HindIII</i> digestion and cloned into pACYC184.	-
pAAG103	Cloning of the <i>SspI-HindIII</i> fragment from nucleotide 1067 to 1754 into the <i>NruI-HindIII</i> site of the vector pACYC184.	+

* Derivatives shown with the ending numeral "45" have the same sequence as the preceding derivative and the 4.5 kb Kan/*oriT*_{RK2} cassette (Hengen and Iyer, 1992b) linked *in vitro* to each of them.

Figure 18. Partial map of pCU1 showing the deletion derivatives from pAAG8 and pAAG5. The numbers indicate the coordinates (kb) of pCU1. Four of the eight *tra* complementation groups are shown as well as the *kikA* and *kikC* region, the presence of the latter being inferred from this study. The region between coordinates 27.7 to 33 has been expanded to show below it in solid black lines the deletion derivatives constructed from pAAG8 and pAAG5. The triangle indicates the Tn5 insertion in pCU171 at coordinate 32.4 (Thatte *et al.*, 1985a). The large open rectangle and the partial rectangle with the letters A and B indicate the *traA* and *traB* complementation group. At the right of each derivative the small squares indicate the region from IS50 Tn5 that remains in the construct. At the left each derivative has in brackets the nucleotide where the deletion ended. The ability of the DNA of each derivative to transform the parental non-immune *Klebsiella* UN2979 is indicated as + under "transformation" to the left.

Transformation

approximately the same proportion. For convenience, this series of experiments and their results is illustrated and summarized as a diagram in Figure 19. Thus, by progressive deletion, a small region of 1,754 bp (as in pAAG5, Figure 18) from within the *traA* region is strongly implicated as being necessary for three phenotypes in *Klebsiella* that may be related: reduction or abolition of transformation activity of DNA, a thousand fold reduction in conjugative mobilization and the ability to give rise to *Klebsiella* that are immune to the killing action of the parental plasmid pCU1 or its derivative pCU109 both of which contain the two previously identified genes *kikA* and *kikB*. This region contains ORF123 and the reading frames to the right of it as shown in Figure 15. The position of the mutations that restored high efficiency mobilization and transformation to pCU1140/pCU11403, pAAG8/pAAG8451 and pAAG7/pAAG7451 are shown in table 10 and Figure 20. Inspection of the sequence of ORF165 (Figure 14) and the sequences present in pAAG6 (Figure 18) indicates that the initiation codon (ATG) is located only 9 bp away from the *Hind*III site in the poly-linker of its vector pUC128. No Shine-Dalgarno sequence is detected and there seems to be insufficient upstream DNA for transcription signals. Nevertheless, and in order to be certain that ORF165 was not involved in these phenotypes the Ω fragment of Prentki and Krisch (1984) containing transcription and translational termination signals was introduced into the *Hind*III site of the pUC128 portion of pAAG6. The resulting plasmid (pAAG5 Ω) was unable to transform *Klebsiella*.

Figure 19. A flow-chart outlining the series of experiments that implicates a region from coordinates 30.7 to 32.4 (nucleotide 1-1754, Figure 18) of pCU1 in the three phenotypes that are considered. Plasmid pAAG7 containing Kan/*oriT*_{KK2} (pAAG74S) is used as an example.

E. coli S17-1 carrying pAAG745

X

parental *Klebsiella* Imm⁻

low frequency (10⁻⁷) of transconjugants

Imm⁻
(88-90%)

plasmid DNA

transformation of

Imm⁻ *Klebsiella*

No detectable
transformants

Imm⁺ *Klebsiella**

large number of
transformants

Imm⁻
(10-12%)

plasmid DNA

transformation of

Imm⁻ *Klebsiella*

large number of
transformants

Imm⁺ *Klebsiella* *

large number of
transformants

*These Imm⁺ *Klebsiella* were derived by introducing pCU1139 into the parental Imm⁻ *Klebsiella* and subsequently isolating a spontaneously-cured derivative.

These results thus implicated the region containing ORF123, ORF63 and ORF101 (Figure 15) as being necessary for at least the phenotypes of lowered mobilization frequency and lack of transformation.

Defining the *kikC* region

To remove sequences extraneous to *kikC*, the region from the *Pst*I site in *traA* to the *Hinc*II site at nucleotide 917 (coordinate 31.5 in the pCU1 map) was deleted from pAAG5 leaving the region 917 to 1754 joined to the vector (Figure 18, pAAG1). After digestion with the enzymes *Pst*I and *Hinc*II the fragments were separated by electrophoresis on a 0.8% agarose gel, and the fragment with the appropriate size of 837 bp expected to contain the *kikC* region, was recovered from the gel. The ends of the molecule were filled in with a mixture of the four dNTPs and the Klenow polymerase and ligated. It was suspected that this DNA containing only the *kikC* region may not yield transformants even in *E. coli*. This suspicion arose from the results of the previous experiments in which the deletion of the *Hpa*I fragment from pCU1107 (in Figure 18, this *Hpa*I fragment extends from a *Hpa*I site in the IS element in *traA* to the *Hpa*I site at coordinate 32.0) gave deleted derivatives with the deletion always larger than expected, extending beyond the *kikC* region suggesting that the removal of this *Hpa*I fragment without the simultaneous removal of the *kikC* region was lethal to *E. coli*. The ligated fragment containing *kikC* was therefore transformed separately into *E. coli* C600 and C600 carrying

Table 10. Description of spontaneous mutants of non-transforming plasmids that have gained transforming ability

Plasmid derivative	Source	Selection	Mutation
pCU11403	pCU1140	Rare <i>Klebsiella</i> transconjugant that is Imm ⁻	Insertion of an IS10-like element at position 1629 in the 112 th codon of ORF123 (see also Figure 10)
pAAG8451	pAAG845	Rare <i>Klebsiella</i> transconjugant that is Imm ⁻	Insertion of an IS1-like element at position 1366 in the 24 th codon of ORF123
pAAG7451	pAAG745	Rare <i>Klebsiella</i> transconjugant that is Imm ⁻	Replacement of thymine (T) for adenine (A) at position 1467 resulting in the replacement of the 58 th codon of ORF123 TGG for AGG (tryptophan for arginine)
pAAG100	pAAG1	Rare transformant of <i>E. coli</i> that also transformed Imm ⁻ and Imm ⁻ <i>Klebsiella</i>	A change of thymine (T) for cytosine (C) at position 1300 that replaced the codon ATA (isoleucine) for ACA (threonine) in the second codon of ORF123 and simultaneously in the sixth position of the -10 sequence of the promoter-like sequence at position 1300

pCU403 (a compatible plasmid carrying the *HpaI* fragment *in trans*). Only the *E. coli* carrying pCU403 gave rise to transformants, a result reminiscent of those observed in pKM101 where a plasmid *kor* (*kil* override) region overrides the effect of a plasmid *kil* gene in *E. coli* (Winans and Walker, 1985a). The additional small plasmid derivative recovered from *E. coli* C600/pCU403 transformants was called pAAG1 (see Figure 18 and 20). It will be confirmed that pAAG1 containing the region called *kikC* is not only unable to transform *E. coli* but is also unable to transform immune or non-immune *Klebsiella*. The gene giving rise to the *E. coli* phenotype is called *kilC*. It could be the same as *kikC'* but a separate designation was used to allow for the possibility that the two genes may be distinguishable. In the rest of this thesis, the region is referred to as *kikC'* or *kilC'* depending upon whether the relevant experiments are in *Klebsiella* or *E. coli*.

Transformants arising in *E. coli* C600/pCU403 were grown in the presence of ampicillin and kanamycin and the two plasmid DNAs were isolated as a mixture in a CsCl preparation. In order to isolate pAAG1 alone, the DNA preparation was separated by electrophoresis in a 0.8% agarose gel. The faster migrating covalently closed DNA band was isolated from the gel and used to transform *E. coli* C600 with and without pCU403. Only the host containing pCU403 gave rise to a large number of transformants. Three rare transformants were isolated from C600. The plasmid DNA isolated from one of these rare transformants (called pAAG100) was

Figure 20. Nucleotide sequence of pAAG1 containing the *kikC/kilC* region. Numbering of the nucleotide sequence begins at the *Hinc*II site (917) of the pAAG8 sequence and ends at the *Hind*III site at position 1,754. Putative promoter-like sequences (-35 and -10) and potential ribosome-binding sites (SD) are underlined. The deduced amino acid sequence is shown and numbered under the sequence. The stop codons are marked by an asterisk. Downward arrowhead indicates the position of IS insertion or point mutations located in the different derivatives and which resulted in the ability to transform *Klebsiella* efficiently (see also Table 10). pAAG1 DNA was unable to transform *Klebsiella* (both the parental strain and the immune derivatives) or *E. coli*. Other symbols are as in Figure 14.

HincII

914 GTCAACCGAAAGACCCAGGCCCACTAAATTATTTGAGTTAATTTGT 960

GCAAGGCCCATATCAAACTTTTATTATCCTTCAGCAGAACTTTTCGCAACGCTGACGGCC 1,020

SspI

TCAGCTTCAGTACGCGGTTGCTGTTTTAACTGAATACTACCGTTAATATTGATCCTGTAC 1,080

-35 -10

GGTCCATTTGAGGACTCATGGCCGACGATGTACGCCATCGTTAAGGGTGAAACATCAGGC 1,140

SD M Y A I V K G E T S G
→ ORF63

GCACATCTCTGCGCAAGCCTGGCAACTTCATCGGAGGCAGGCGCACGACTGGCACGGCCA 1,200

A H L C A S L A T S S E A G A R L A R P

GCACAAGCAAGGCAGGCCAGAGCGAGAACCAGGAGTTTTGGATGTTTACTCATTATGTT 1,260

A Q A R Q A R A R T R S F G C L L I Y V

▼

TCACCGCGTAATTGTAATTTGACGACTTTTGTTTATGATAAGGGCTAATGAGATCGTGT 1,320

-35 -10

S P R N C N F D D F C L * *

M I R A N E I V
→ ORF123

▼

CAAGAAGAAACGAAAAGAAAACCGCTGAAGGAAGGAAGGTGATGCTGGTTCCCCTGAAAT 1,380

SD

S R R N E K K T A E G R K V M L V P L K

→ → ORF101

CAGAAAAACGACCAAAAGGCGAACCCGTGTATCGTGACCCGGATAACCCTTTTAATACGT 1,440

S E K R P K G E P V Y R D P D N P F N T

▼

GGACTGGTATAGGGAAGCGCCCGGCTGGCTAACTGCAAAATTGGACGCTGGCATTAGCC 1,500

W T G I G K R P A W L T A K L D A G I S

TGGAAGCCATGAAGATGCAGGGCGTAGCCAACCCAGAGAACATAGACCAGCAAAATACC 1,560

L E A M K M Q G V A N P R E H R P A K Y

GCGACCCAGGAACGCAGAAAATACCTGGTCCGGGACTGGCCGCCGACCCACATGGCTCA 1,620

R D P R N A E N T W S G T G R R P T W L

▼

AAGAGCTGCTTGATAGTGGTTTATCACTTGATGATCTGAAGATATAACCGGAGGGTATAA 1,680

K E L L D S G L S L D D L K I *

AAATGGGCGAACTTATTGATTTTTCGGAAAGGCAAAAAAGCAGAAGGAAGAAAAAGCAT 1,740

CTTCATTCCGCCCAAGCTT 1,754

HindIII

used to transform Imm⁻ and Imm⁻ *Klebsiella* and the Imm⁺ *Klebsiella* containing pCU403. The results were compared to transformation with pAAG1 and pAAG5. They are shown in Table 11. pAAG1 that was not able to transform *E. coli* without pCU403 was also not able to transform either of the *Klebsiella* strains (Imm⁻ or Imm⁺) unless pCU403 was provided. Thus, immunity to the Kik⁺ phenotype (no loss of viability upon mating with C600/pCU1) is insufficient to afford protection from the lethal effect of *kikC* in pAAG1. In contrast to pAAG1, pAAG5 transformants are obtained in immune *Klebsiella* without pCU403 (see Table 11). This result indicates that the region present in pCU403 that provides protection against *kikC* and *kilC* is also present in pAAG5. It restricts the protective region of pAAG5, designated as *korC* (for killing override of *kikC* or *kilC*) and excludes the need of other regions present in pCU403 for such protection in *E. coli* or in *Klebsiella* that have been made immune.

Nucleotide sequence of the *kikC* region and the position and nature of plasmid mutations that restore transformation ability.

The nucleotide sequence and the predicted amino acid sequence of the three potential ORFs present in the *kikC* region (ORF63, ORF123 and ORF101) is shown in Figure 20. Also shown in this Figure, is the position of all spontaneous plasmid mutations (of pAAG1 or larger derivatives) that restored transformability to each plasmid. With the exception of pAAG100 all other plasmid mutants were isolated as

Table 11. Transformation of Imm⁻ and Imm⁺ *Klebsiella oxytoca* strains by plasmid pAAG5, pAAG1 and pAAG100

Plasmid	<i>Klebsiella oxytoca</i>		
	UN2979	UNC1139	UN2979/pCU403
	Imm ⁻	Imm ⁺	Imm ⁺
pAAG5	-	+	+
pAAG1	-	-	+
pAAG100	+	+	+

+, indicates $> 10^3$ transformants

-, indicates no transformants

The same preparation of competent cells was used for transformation with 1 μ g/ μ l of each plasmid DNA.

rare *Klebsiella* transconjugants with mobilizable derivatives carrying the linked *Kan/oriT_{RK2}* cassette. Their origin and description is summarized in Table 10. No chromosomal mutants were isolated. The first three of the mutations listed in Table 10 are at a position that includes ORF123 and ORF101. In the fourth mutant, while a change could have been introduced in the second amino acid of ORF123, it could also have resulted in the disruption of activity of a putative promoter for ORF101. The comparative sequence analysis of 112 *E. coli* promoters compiled by Hawley and McClure (1983) indicated that the most highly conserved base in the -10 region of the promoter is a T at the sixth position which is exactly the base that has changed in pAAG100. Figure 21 shows the change in pAAG100, as compared to the wild type sequence.

The promoter located before ORF63 could be translating the four possible products ORF101, ORF63, ORF123, or a possible combination of ORF63 and 123. On the other hand, the promoter before ORF101 could transcribe only this product. To determine which of these promoters is relevant to the activity of *kikC* or *kilC*, the following experiments were done. The -10 sequence of the potential promoter of ORF63 which is located at position 1,067 contains a *SspI* site (Figure 20). Cloning the *SspI*-*HindIII* fragment into the *NruI*-*HindIII* site of pACYC184 is therefore likely to inhibit the promoter upstream of ORF63 but not the one upstream of ORF101. After this cloning, transformants were isolated in *E. coli* C600 containing pCU403 on LE plates with chloramphenicol. The derivative denoted pAAG103 was

Figure 21. Autoradiograms of a portion of the sequencing gels showing the sequence comparison of pAAG1 and the mutant pAAG100. Left panel shows the sequence of the pAAG1 region where a mutation was found in pAAG100, shown in the right panel. The arrows indicate the change in the nucleotide. The letters G, A, T and C above the respective lanes indicate the dideoxynucleotide used in the sequencing reaction.

isolated from the mixture and its DNA was used to transform the strains C600, and the Imm⁻ and Imm⁺ *Klebsiella*. All three recipients were transformed efficiently. This results suggests that the putative pCU101 promoter is not involved in the expression of the KikC or KilC phenotypes and also excludes the possible transcription of ORF101 from this promoter. The possibility that ORF101 is initiated within a transcript initiated from the promoter located before ORF63 is an open one.

Table 10 indicates that two of the four mutations in the *kikC* (*kilC* region) are caused by insertion elements referred to as IS10-like and IS1-like. These are well characterized insertion elements discovered and described in *Salmonella typhimurium* and in *E. coli* (Galas and Chandler, 1989). The insertion element referred to as IS10-like has the same size as IS10 of the transposon Tn10 (see Figure 11). The *Klebsiella* insertion element was not sequenced in its entirety but when 65 bases of it were sequenced and searched, it indicated a 100% homology to IS10. Similar conclusions were reached for the second mutation in which 56 bp were sequenced and had 100% homology to the 5' end of IS1. As far as I am aware, this is the first observation of IS1 and IS10-like sequences in this well-known strain of *Klebsiella oxytoca*.

Defining the *kor* region for *kikC* (*kilC*)

It was shown that plasmid pAAG5 (Figure 18) can be conjugatively mobilized into both *E.coli* and *Klebsiella* and that it can also be transferred into

immune *Klebsiella*. However, it was shown in the previous section that it contains a sequence with potential lethal effects (*kikC/kilC*; nucleotide 917 to 1754); therefore, it can be concluded that the additional presence of the region between nucleotides 1 to 917 might be controlling the expression of *kikC/kilC* or neutralizing their lethality. In order to determine whether this second region called *korC* could work *in trans* to *kilC* in *E. coli*, the plasmid derivatives pAAG10 and pAAG15 (see Table 9 and Figure 18) were transformed into *E. coli* selecting the transformants on ampicillin plates. The resulting *E. coli* containing either pAAG10 or pAAG15 were then transformed with pAAG1-184 selecting for chloramphenicol resistance. Transformants were recovered at a high frequency, similar to the transformation frequency observed with the vector pACYC184. pAAG1-184 was constructed by retrieving the *kikC* (*kiC*) region present in pAAG1 using the enzymes *Bam*HI and *Hind*III and cloning the fragment into the *Bam*HI-*Hind*III sites of the vector plasmid pACYC184 and selecting Cm^r, Tc^r transformants. This derivative of pAAG1 was made to provide a *kikC* (*kilC*)-carrying plasmid that would be compatible with the *kor*-carrying pUC vector plasmids derived from pAAG5 (ie. pAAG10 and pAAG15). In order to analyze if the same region could also be protecting *Klebsiella* as considered before, the two plasmid derivatives pAAG10 and pAAG15 were introduced by transformation into Imm⁻ and Imm⁺ *Klebsiella* cells. Transformants were selected on ampicillin plates. The constructed derivatives were then used in transformation experiments with pAAG1-184. The results indicated that only Imm⁺

Klebsiella could give rise to transformants comparable in number to those obtained with the plasmid pACYC184 (approximately 10^4). Only a few transformants were isolated with the Imm⁻ *Klebsiella* and they have not been analyzed further. I presume that these rare pAAG1-184 transformants might be mutants, in which *kikC*' function has been inactivated (similar to pAAG100).

From these experiments it could be concluded that the *korC*' region is located between nucleotide 1 to 630 (*Nar* site in pAAG15, Fig 18) and that it can function *in trans* in *E. coli* and also in *Klebsiella*, provided that *Klebsiella* carries the chromosomal mutation that confers the immune phenotype. This result confirms the results obtained with pAAG5 when *kor* was provided in *cis*.

The *kor* region present in pAAG15 (nucleotide 1 to 630) contains four possible ORFs, ORF115, 62, 93 and 73 already described in Figures 14 and 15. The plasmid DNA from pAAG15 was digested with the enzyme *NarI*, opening the plasmid at nucleotide 630, and the DNA was treated with the exonuclease *Bal31* in order to generate deletion derivatives that could indicate the orientation of transcription of the *kor* gene as well as the ORFs involved in the phenotype. The putative promoter for ORF93 (the -35 sequence) is located at position 574 to 602 and the *NarI* site is located at position 630; it was reasoned that treatment with *Bal31* might inactivate this putative promoter without affecting the ORFs transcribed from the upper strand (the one reading towards *kikA*) containing ORF115 and ORF62. After *Bal31* treatment, the DNA was filled in, ligated and used to transform

DH5 α competent cells. Transformants from each one of the *Bal*31 treatments were purified and their plasmid DNA was isolated and analyzed for the extent of the deletion; the product of one of the digestions yielded a derivative that seemed to provide only partial protection to the *E. coli* strain when it was transformed with the derivative pAAG1-184. Transformants arose on chloramphenicol plates only after 48 h when they were tiny, continuing to develop only slowly during further incubation. This derivative was called pAAG15-13 and its DNA was sequenced. The extent of the deletion is shown below.

Wild type pAAG15

590	600	610	620	630 (NarI site)
CAT CACCAGAACC AAAACATCT TGTTCAACCGT CATACTGCGG				
GTA GTGGTCTTGG <u>TTTTTGTACA</u> ACAAGTGGCA GTATGACGCC				
-35				

*Bal*31 derivative pAAG15-1

590	600
CAT CACCAGAACC AGTTC	
GTA GTGGTCTTGG <u>TCAAG</u>	
-35	

The sequences described above indicated that the *Bal*31 treatment giving rise to pAAG15-13 removed the nucleotides from 630 to 601 and the ligation produced a new sequence at the -35 position. This result indicated that the protection against

kilC is most likely provided by the ORFs transcribed from this promoter. The appearance of tiny and slowly developing colonies seems to indicate that the new -35 sequence might still be functional in providing weak protection.

Is the *kikC* region sufficient for giving rise to immune *Klebsiella*?

To answer this question the plasmid derivatives pAAG445, pAAG345 and pAAG245 (see Table 9) containing the *oriT_{RK2}* were transformed into S17-1 and the purified transformants then used in mating experiments with UN2979Rif^r *Klebsiella* recipients. Transconjugants were selected on kanamycin and rifampicin plates. The plasmid pAAG4 (nucleotide 1 to 1597, Figure 22) contains truncated ORF123 and ORF101 (with sequences determining 23 amino acids missing from their C termini), and the entire *kor* region; the plasmid pAAG3 (nucleotide 1-1390, Figure 22) contains ORF63 and a truncated ORF123 and ORF101 (in this case only 31 amino acids of ORF123 and 10 amino acids of ORF101 from their respective N termini); it also contains the entire *korC*. The plasmid pAAG2 (nucleotide 1 to 1158, Figure 22) does not contain *kikC(kilC)* but has the *korC* region. The frequency of conjugative transfer with all three derivatives was high, from 8 to 10%. Thus, these derivatives do not reduce the viability of the recipient. 50 pAAG445 *Klebsiella* transconjugants, 25 transconjugants from pAAG345 and 50 from pAAG245 matings were analyzed for the immune phenotype as described in Materials and Methods. Imm⁺ transconjugants were recovered at a frequency of 88% while the Imm⁻

Figure 22. Physical location of *kikC/kilC* and *korC*, indicating the ORFs present in the deletion derivatives that defined the regions. The top bar shows the coordinates of the parental pCU1 map, the second bar kilobases from the unique *Pst*I site in pAAG5. The relevant ORFs are shown with arrows indicating their direction and their inferred start and stop positions. The lines below the ORFs represent the deletion derivatives that were used to define the two regions; the end point of the deletion is shown at the left.

transconjugants represented 12% of the population from matings when pAAG445 and pAAG345 were used as donors. The plasmid was cured from some of the immune transconjugants (as determined by the loss of plasmid markers) and they continued to be immune after losing the plasmid. Transconjugants from the matings with pAAG245 were all Imm⁺. These results dissociate the ability of plasmid derivatives to give rise to immune *Klebsiella* from their inability to give rise to transformants or transconjugants at a high frequency. They indicate that deletion of the C-terminal region of ORF123 and ORF101 inactivates the *kikC* phenotype but does not affect the ability of the derivatives to give rise to immune *Klebsiella*. The results obtained with pAAG345 reduce the region inducing the immune phenotype in *Klebsiella* to one that contains ORF63, the truncated ORF123 and ORF101 on the upper strand and ORF234 on the lower strand in addition to the sequences present in the *kor* region (Figure 22). On the other hand, pAAG2 or pAAG245 in which all of ORF123, ORF101, ORF63 and ORF234 are deleted (see Figure 22 and Table 9), do not give rise to immune *Klebsiella*. Neither does pAAG15 which, like pAAG2, carries only the *korC* region (nucleotide 1-630).

These results taken together seem to indicate that the region comprising *korC*, the VirB1-like ORF234, and part of the *kikC* region (although the lethal effect of this region is inactivated) is sufficient for giving rise to immune *Klebsiella*. The observation that *Klebsiella* carrying only pAAG15 (the *korC*-containing plasmid) cannot be transformed with pAAG1-184 carrying *kikC* alone, suggests that for

immune *Klebsiella* to arise, either *kikC* and *korC* must be present *in cis* to each other or that the region between them must be present (see Figure 22). Since a derivative with partially deleted and functionally inactive *kikC*, gave rise to immune *Klebsiella* the latter alternative is favoured. Further experiments will be needed to determine whether both the VirB1-like ORF234 and *korC* are both needed or whether ORF234 alone will suffice to give rise to the Imm⁺ phenotype.

Results Part 2
The *kikB* region

The *kikB* region has been located at the end of *tra* close to *oriT* (Thatte *et al.*, 1985b). It was defined on the basis of a comparison of the *Kik*⁺ phenotype conferred by pCU109 a *Kik*⁺ derivative and the *Kik*⁻ or partially *Kik*⁺ derivative, pCU101. The region of pCU1 present in pCU101 and pCU109 derivatives was shown in Figure 1. Plasmid pCU109 was constructed from pCU88, a Tn5 insertion in pCU1 located at coordinate 10.2 as described in the first part of the result section. pCU101 was constructed in a similar manner, by cloning the *Hind*III fragment containing the transfer region from coordinate 27.7 to coordinate 8.7. Therefore, the *kikB* region is inferred to be located between coordinates 8.7 and 10.2. These coordinates are somewhat different from the one presented by Thatte *et al* (1985b) based on restriction mapping. The data presented here is based on sequence analysis from the *Sma*I site at coordinate 1.8 in the pCU1 map, to the site of the Tn5 insertion in pCU101 at coordinate 8.7. The region from coordinate 8.7 to 10.2 was sequenced in this work using for this purpose several plasmid derivatives that will be described later. Thatte *et al* (1985b) had left open the possibility that *kikB* may only be a small part of the region defined by the difference between pCU109 and pCU101, because 33 Tn5 insertions in the vicinity of *kikB* (pSV derivatives; Thatte, 1985) did not affect the *Kik*⁺ phenotype.

I first determined the transfer frequencies of pCU109, pCU101 and their parental plasmids pCU88 and pCU29 from rifampicin-sensitive *E. coli*, to *E. coli* HB101Rif^r and *Klebsiella oxytoca* UN297SRif^r recipients. The frequencies were

compared to the wild type plasmid pCU1 and to the plasmid pCU56 which contains the *kikB* region cloned onto the vector plasmid pACYC184. As pCU56 is not conjugatively self-transmissible, it was transferred by being mobilized by pCU801, which is itself not transferred. The results shown in Table 12, indicated that the Tn5 derivatives pCU88 and pCU29 had a frequency of transfer comparable to pCU1 when they were transferred to *E. coli* and to *Klebsiella* recipients. On the other hand, the frequency of transfer of pCU109 and pCU101 to *Klebsiella* was a 100 fold less than the parental plasmids pCU88, pCU29 and pCU1 even though their frequencies of transfer to *E. coli* were comparable to one another.

One of the requirements to detect the Kik⁺ phenotype is to have efficient mating; conditions that reduce or abolish proficient mating of plasmid pCU1 also reduce or inhibit the Kik⁺ phenotype (Rodríguez and Iyer, 1981). Therefore, the killing ability is an indirect way of detecting efficient transfer of the plasmid to a *Klebsiella* host. The killing efficiency of pCU109 was comparable to pCU1 (Thatte *et al.*, 1985b), but its recovery in *Klebsiella* was 100 fold less than that of pCU1. This result suggests that transfer *per se* is not sufficient to explain the reduction in the number of transconjugants and that the region missing in pCU109 and present in pCU1 (from coordinate 10.2 to 27.7 clockwise in the pCU1 map) might specify important functions that help the establishment of the plasmid when *Klebsiella* is the recipient.

Table 12. Comparison of the conjugative transfer of pCU1 and its derivatives to *E. coli*, Imm⁻ and Imm⁺ *Klebsiella*.

Plasmid in C600 <i>E. coli</i> donor	Selected marker for transfer	Frequency of transfer* to		
		<i>E. coli</i>	UN2979	UNC109
pCU1	Sp	2.0×10^{-1}	2.0×10^{-4}	4.0×10^{-1}
pCU88	Km	1.5×10^{-1}	2.0×10^{-4}	3.5×10^{-1}
pCU109	Cm	1.4×10^{-1}	5.0×10^{-7}	3.0×10^{-1}
pCU29	Km	1.4×10^{-1}	1.0×10^{-4}	3.4×10^{-1}
pCU101	Cm	1.5×10^{-1}	1.0×10^{-7}	3.0×10^{-1}
pCU56 mobilized by pCU801	Cm	2.0×10^{-1}	2.2×10^{-1}	3.0×10^{-1}

* A donor to recipient ratio of 1:10 was used to determine the transfer frequency on membrane filter for 1 h at 37°C. The numbers represent the frequency of transfer and it is the average of two experiments.

The analysis of pCU101 transconjugants was more surprising; a Kik⁻ or Kik^{+/+} derivative like pCU101 is expected to have a higher frequency of transfer than pCU1 due to the lack of inhibition of the host but this was not the case. On the contrary, the frequency of transfer of pCU101 to *Klebsiella* was low. The difference in recovering transconjugants after mating might reflect a problem in the establishment of this plasmid in *Klebsiella*. If the vector pACYC184 is inherently unstable when it is connected to the transfer region of pCU1, the effect may be expected to be seen in *E. coli* as well as *Klebsiella* hosts. This is not the case; the effect is observed only in the parental non-immune *Klebsiella*. Table 12 with pCU101 also shows the frequency of mobilization of pCU56 containing the *kikB*, *oriT* and part of the *tra* regions of pCU1 cloned into the *Bam*HI site of pACYC184. This construct exhibits the same frequency of transfer to *E. coli* and to *Klebsiella* when mobilized by the helper plasmid pCU801 indicating that the *traH* to *kikB* region is neither lethal to the *Klebsiella* nor sufficient to reduce the transconjugation frequency.

What makes pCU101 a Kik⁻ or Kik^{+/+} derivative ?

Analysis of the restriction pattern of pCU109 and pCU101 DNA isolated from purified *Klebsiella* transconjugants indicated that transconjugants from pCU109 had the expected restriction pattern (data not shown); but in the case of pCU101 transconjugants, the pattern of the DNA digested with the enzyme *Hpa*I was

Figure 23. Agarose gel of plasmid DNA isolated from *Klebsiella* and *E. coli* transconjugants of pCU101, digested with *Hpa*I. Hybridization pattern of the gel with ³²P labelled *kikA* region.

Panel A: Lane A, B, C, D, E plasmid DNA isolated from independent UN2979/pCU101 derivatives (10 derivatives were analyzed of which only 5 are shown)

Lane F. Plasmid DNA from HB101/pCU101 used as control

Lane G. Molecular weight standard, λ DNA digested with *Hind*III restriction enzyme. Fragment size from top to bottom are in kilobases 23.1, 9.4, 6.5, 4.3, 2.3, 2.2. The fragment of 0.5 is not visible. The positions are indicated by arrowheads.

Panel B. Southern blot hybridization of DNA shown in panel A, transferred to a nylon membrane and hybridized with ³²P *kikA* fragment (0.5 kb) isolated from pPH4. Only the plasmid with the wild type pCU101 restriction pattern hybridized with the probe (lanes E and F).

A B C D E F

rearranged in nine out of ten of the transconjugants analyzed. Only one of the six *Hpa*I fragments present in pCU101 was present in the derivatives indicating that large deletions had occurred. The gel pattern of some of the deleted derivatives are shown in Figure 23, as well as the gel pattern of one transconjugant that did not rearrange and had the wild type restriction pattern. Figure 23A also shows the pCU101 DNA pattern of one transconjugant isolated from an *E. coli* host that was used as a control. Figure 23B, shows the hybridization of the DNA present in the gel described in Figure 23A, with a ³²P labelled probe containing the *kikA* region. Only the transconjugants showing the wild type restriction pattern hybridized with the *kikA* probe (lane E, UN2979/pCU101 and lane F, HB101/pCU101). The results presented here seem to indicate that the *kikB* region when present with the rest of the DNA present in pCU101 might be playing a role in the stabilization or establishment of the plasmid but only when *Klebsiella* is the host. Figure 24 shows the restriction pattern of independent transconjugants isolated from a mating of C600/pCU101 as the donor and immune *Klebsiella* UNC109 transconjugants. The gel pattern of the transconjugants isolated from Imm⁺ *Klebsiella* (lane A, B, C, and D) was similar to the wild type DNA isolated from *E. coli* transconjugant (lane E). This result indicates that immunity which is believed to be a chromosomal change in the *Klebsiella* chromosome (results. Part I) contributes to stabilize the plasmid in such a manner that rearrangements do not occur.

Figure 24. Agarose gel of plasmid DNA isolated from *Klebsiella* UNC109/pCU101 and *E. coli* HB101/pCU101 digested with the restriction enzyme *Hpa*I.

Lane A, B, C, D. Plasmid DNA isolated from UNC109/pCU101 transconjugants

Lane E. Plasmid DNA isolated from HB101/pCU101 transconjugant used as control

Lane F. 1 kb DNA ladder.

A B C D E F

While studying the *kikB* region, Thatte *et al* (1985b) have made an important observation; the Kik⁺ phenotype of pCU101 was restored when it was ligated to pCU51. This derivative carried the pCU1 region from coordinates 8.7 to 21 which includes the *kikB* region and the replicon of pCU1 (see Figure 25). The cointegrate plasmid pCU114 displayed the Kik⁺ phenotype. The authors also showed that when the *kikB* region was cloned in a plasmid compatible with pCU101 (like pCU404) and both plasmids (pCU101 and pCU404) entered the *Klebsiella* recipient at the same time during mating, the killing phenotype was restored. On the contrary if pCU404 was first established in *Klebsiella*, and pCU101 transferred to it subsequently, the Kik⁺ phenotype was not detected; when a purified transconjugant from the latter mating was used as donors in a second mating experiment, the derivative was able to display a Kik⁺ phenotype against a nalidixic acid-resistant isogenic *Klebsiella*. They concluded that in order to express Kik⁺, the *kikA* region present in pCU101 and the *kikB* region present in pCU404 must be transferred simultaneously to *Klebsiella* and that the Kik⁺ phenotype is expressed by the joint action of the two regions.

The Tn5 insertion in pCU29 (coordinate 8.7) that gave rise to pCU101 was located 350 bp away from the *oriT* region; therefore the *kikB* region (coordinates 8.7 to 10.2) must be part of a region known as the "leading region" of the plasmid, which in several groups of plasmids is the name given to the first segment of DNA to be transferred into the recipient cell during conjugal transfer. Several genes

Figure 25. Diagram illustrating the methods used for the construction of plasmid derivatives from pCU29, a Tn5 insertion derivative of pCU1 (shown as a triangle). pCU51 is a *Bam*HI deletion derivative of pCU29, and the cloning of the transfer region from pCU29 into the vector pACYC184 gave rise to pCU101. Plasmid pCU101, a Kik⁻ derivative can restore a Kik⁺ phenotype by forming a cointegrate with pCU51 as in pCU114 or by the help of pCU404 (a derivative containing the *kikB* region cloned in the vector pMK2004) when both are transferred simultaneously from the *E. coli* donor to the *Klebsiella* recipient. *kikA* and *kikB* are the regions required for the killing of *Klebsiella*; *bom* is the region required in *cis* for plasmid transfer; Cm, chloramphenicol; Km, kanamycin; B, *Bam*HI, K, *Kpn*I; H, *Hind*III. Courtesy of Thatte V. (Thatte *et al.*, 1985b).

involved in plasmid establishment or stability have been located in the leading region of the well known F plasmid and its close relatives. The leading region of the F plasmid also shares homology with unrelated plasmids (Golub and Low, 1986). The conservation of the region among the group of conjugative plasmids was interpreted as having an important role in conjugation and survival of the plasmid. The *kikB* region may therefore be specifying some function that helps establishment of the plasmid after it has been transferred. The interesting point here is that it is host specific.

***kikB* region and the immune phenotype**

As mentioned in the first part of results, the *kikB* region present in pCU56 is not able to induce the immune phenotype; the transconjugants from pCU109 matings were Imm⁺ but the transconjugants from pCU101 matings were Imm⁻, this result was interpreted by Gill (1985) as an indication of the requirement of *kikB* and *kikA* regions to express or induce the immune phenotype in *Klebsiella*. In this work it was observed that the rare *Klebsiella* transconjugant from pCU101 matings that conserved the wild type restriction pattern was expressing a Imm⁺ phenotype even after loosing the plasmid; therefore, the inability to express the immune phenotype in the majority of the transconjugants in pCU101 matings is correlated with the rearrangements and the formation of deleted derivatives. I presume that the deletion

observed in the Imm⁺ transconjugants removed the pCU1 region that is able to give rise to Imm⁺ *Klebsiella* as defined in the results, Part A.

Nucleotide sequence analysis of the *kikB* region

The nucleotide sequence of the *kikB* region of pCU1 from coordinate 8.7 to 10.2 was determined using two plasmid derivatives, pCU109 (already described) and pCU105, a Tn5 deletion derivative from pCU231 whose Tn5 insertion was mapped between pCU109 and pCU101 at coordinate 9.4 (new location, determined by sequencing) in the pCU1 map. pCU105 contains the transfer region of pCU1 from coordinate 27.7 (*Hind*III site) to 9.4 (*Hind*III site in the Tn5 insertion present in pCU231) cloned into the *Hind*III site of the vector plasmid pACYC184. pCU109 and pCU105 plasmid DNAs were used to sequence the region from the IS element towards the *oriT* region, using the primer complementary to the end of the IS50 sequence.

To facilitate the sequencing strategy the plasmid pCU51 was used to sequence from the opposite direction, using the IS50 primer. I also constructed a clone containing the small *Hpa*I fragment from pCU109 (one *Hpa*I site is located at coordinate 9.5 and the other at position 10.2 (at the end of the Tn5 insertion that gave rise to pCU109), the *Hpa*I fragment was cloned into the *Sma*I site of pUC128 to give rise to plasmid pFSG1. Sequencing was performed on this construct using the M13 reverse and M13 forward primers complementary to the multicloning site

of pUC128. Synthetic oligonucleotide primers were used to complete the sequence on both strands. The complete sequence is shown in Figure 26. Nucleotide 1 corresponds to coordinate 8.7 and nucleotide 1448 to coordinate 10.2 in the pCU1 map. Coordinates for the location of recognition sites for restriction endonucleases with one or two sites in the sequence are described in Table 13.

The *kikB* region was examined in both directions for possible protein coding genes. Potential ORFs were identified by localization of probable initiation codons (ATG or GTG) which were preceded by reasonable ribosome binding sites (Shine-Dalgarno sequence) and terminated with a stop codon. Two ORFs were found in the sequence reading towards *oriT* and four in the opposite direction. They are shown in Figure 27. Table 14 lists coordinates for their translational start and stop codons and also the molecular weight of these predicted polypeptides. A promoter-like sequence was located at position 137 to 166. The -35 hexanucleotide matches the consensus *E. coli* -35 hexanucleotide TTGACA with a A+T rich hexamer corresponding to -10 position of the promoter located 18 nucleotides downstream. The sequence GGAG found within the Shine-Dalgarno sequence (Shine and Dalgarno, 1974) was located 6 nucleotides upstream of ORF75 and 7 nucleotides upstream of ORF100. The Shine-Dalgarno like sequences upstream of ORF125, 50 and in the opposite direction, the two ORF60s, do not have a perfect match. ORF75 from nucleotide 591 to 811 has two internal ATG sites located at position 624 and 675. ORF51 and ORF60 (closer to *oriT*) has a GTG initiation site.

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Figure 26. Nucleotide sequence of the *kikB* region. The numbers indicate nucleotide position with nucleotide one corresponding to coordinate 8.7 and nucleotide 1448 to coordinate 10.2 in the pCU1 map (see Figure 1). Putative promoter sequences (-35 and -10) and putative Shine-Dalgarno sequences (SD) are underlined. Rightward arrows indicate ORFs toward coordinate 10.2, leftward arrows indicate ORFs with the orientation towards *oriT*. Initiation codons are represented in bold. Stop codons for the ORFs in the upper strand are represented by an asterisk (*) and the ones in the lower strand by the downward arrow (↓).

10	20	30	40	50	60
CTGGGGATTG	AGAATGTTTC	CATTTTAAA	GGTGATGACT	TTTCACGGTT	GATTGAAGAT
GACCCCTAAC	TCTTACAAAG	GTAAAAATTT	CCACTACTGA	AAAGTGCCAA	CTAACTTCTA
70	80	90	100	110	120
ATTGTTTTTG	AAGATGCCGG	GATTATTGAT	ATTGGTGCGT	CAAACGTAGA	AGCSTTCCTG
TAACAAAAAC	TTCTACGGCC	CTAATAACTA	TAA [↓] CCACGCA	GTTTGCATCT	TCGCAAGGAC
130	140	150	160	170	180
ATGGCAATGT	CCCGCTTTGA	CAGTGGCGCG	AATGAATTTG	ATAAGTATGT	AATCCCGGTT
	-35			-10	
TACCGTTACA	GGGCGAAACT	GTCACCGCGC	TTACTTAAAC	TATTCATACA	TTAGGGCCAA
190	200	210	220	230	240
ACGTCCTGAT	AATAAGGCCA	TTGATGAAAG	CATGAAAACA	GCACATACGT	TAAGTAAGGC
	SD		→ ORF125	↓	
TGCAGGACTA	TTATTCCGGT	AACTACTTTC	GTACTTTTGT	CGTGTATGCA	ATTCATTCCG
250	260	270	280	290	300
AGGTGTAAGC	AGCGATAAAA	TCATCTTTGT	TCCAAACCGC	ATTAGTCCAG	ATAGTGAAGT
			ORF60 ←		
TCCACATTCG	TCGCTATTTT	AGTAGAAACA	AGGTTTGGCG	TAATCAGGTC	TATCACTTCA
310	320	330	340	350	360
AGAGGATGTA	CTGGCGCCGG	TATTTGAATT	TGTCAAACGA	ACGAAAATTG	GCAAAATCAG
TCTCCTACAT	GACCGCGGCC	ATAAACTTAA	ACAGTTTGCT	TGCTTTTAAC	CGTTTTAGTC
370	380	390	400	410	420
CAAGAAATCT	GTTATTTATA	ACAGTGAAGT	TTTCGAATAT	CTCGCGTATC	ACCGTATCTC
GTTCTTTAGA	CAATAAATAT	TGTCACCTCA	AAAGCTTATA	GAGCGCATAG	TGGCATAGAG
				ORF60 ←	SD
430	440	450	460	470	480
ATTGGAAGCA	TTGACCGGTC	TAGATCCAGA	AGAATTTAAA	GCCCCGCGCTA	AACAAACAAC
TAAGCTTCGT	AACTGGCGAC	TTCTAGGTCT	TCTTAAATTT	CGGGCGCGAT	TTGTTTGTG

430	500	510	520	530	540
GGATGCTGAC	GAGCGCAAAA	AACTGGCCCCG	CCGTTATACC	TTATGAAAC	AGGCAATTCC
CCTACGACTG	CTCGCGTTTT	TTGACCGGGC	GGCAATATGG	ATGTACTTTG	TCCGTTAAGG
550	560	570	580	590	600
TGTTAAAGCT	AATCTCGATA	AAGCATATGC	GGCTTTAATG	<u>GGAGAATAAA</u>	ATGGAAAAGC
ACAATTTCTG	TTAGAGCTAT	TTCGTATACG	CCGAAATTAC	SD * → ORF75	CCTCTTATTT
					TACCTTTTCTG
610	620	630	640	650	660
AGCCAGATAA	ATTTGAAGT	CTGATGGATT	GGTTTTTAGG	TGACGCGAAG	GAAATCACCG
TCGGTCTATT	TAAACTTCAA	GACTACCTAA	CCAAAAATCC	ACTGCGCTTC	CTTTAGTGGC
670	680	690	700	710	720
CAAGTCAGAA	AGAAATGACT	GAGATACTTT	CTGCGCTTTC	GGAAAAGCTG	GCAAAAGACA
G TTCAGTCTT	TCTTTACTGA	CTCTATGAAA	GACGCGAAAG	CCTTTTCGAC	CGTTTTCTGT
730	740	750	760	770	780
CCGAAAGTTT	AGGAGAGACG	GCAGACTCTC	TTAAACGGAC	TTTAGTAGAA	AACCAGCGTT
GGCTTTCAAA	TCCTCTCTGC	CGTCTGAGAG	AATTTGCCTG	AAATCATCTT	TTGGTCGCAA
790	800	810	820	830	840
CAATTAGCCT	<u>GGCAATTAGT</u>	GATGATGCTA	AGGCGTGAGG	AATTTCTGAC	GAAGTTCCGC
GTTAATCGGA	SD → ORF51 *	CTACTACGAT	TCCGCACTCC	TTAAAGACTG	CTTCAAGGCG
850	860	870	880	890	900
CGCGGCCAGG	GCTCCAGAGC	TGAGACGTTA	ACCCGTCAGA	TCCTTTTAT	TACAGCTGGC
GCGCCGGTCC	CGAGGTCTCG	ACTCTGCAAT	TGGGCAGTCT	AGGAAAAATA	ATGTCGACCG
910	920	930	940	950	960
TGCACTATTG	TGGGCGCCGC	CGTGGGTGCC	GCGATAGCCA	TAATTCTACT	GAGATAATGT
ACGTGATAAC	ACCCGCGGCG	GCACCCACGG	CGCTATCGGT	ATTAAGATGA	CTCTATTACA

970	980	990	1000	1010	1020
AAACCGGGCA	TGTCCCGGTT	TTTTTCAAG	CGAAGCGCGG	AGGCCGCAGG	CCGGAGGCAT
TTTGGCCCGT	ACAGGGCCAA	AAAAAAGTTC	GCTTCGCGCC	TCCGGCGTCC	GGCCTCCGTA
1030	1040	1050	1060	1070	1080
TAGTGGCCGC	CGCCCGAAGG	GGCGAGAGCG	TAGCGGCTCG	ATGCGCAGCA	CGGCAGAACG
ATCACC GGCG	GCGGGCTTCC	CCGCTCTCGC	ATCGCCGAGC	TACGCGTCGT	GCCGTCTTGC
1090	1100	1110	1120	1130	1140
GCCCCGCAGG	GGTAATGCCC	AGTTTAATTC	AACGTGACAG	TCACGTGGAG	GAAAAATATG
CGGGGCGTCC	CCATTACGGG	TCAAATTAAG	TTGCACTGTC	AGTGCACCTC	CTTTTTATAC
				SD	→
1150	1160	1170	1180	1190	1200
AATGACCGAC	AGCGTGAACA	AGCCCGTATT	CGCCAGGCCC	GGCGCCGCGC	GACTCAAAGA
→ ORF100					
TTACTGGCTG	TCGCACTTGT	TCGGGCATAA	GCGGTCCGGG	CCGCGGCGCG	CTGAGTTTCT
1210	1220	1230	1240	1250	1260
GGAAGGCGCT	AGCTGACAGT	CACGCTAACA	AAACAGGAAG	AAGCAATGTT	ACAGGAGCTT
CCTTCCGCGA	TCGACTGTCA	GTGCGATTGT	TTTGTCCCTC	TTCGTTACAA	TGTCCTCGAA
1270	1280	1290	1300	1310	1320
TGCCGGGTTC	GCCGTCCAGG	ACGAACAGCC	TATTCAACGA	ATGAGTTTTT	CCAAC TGCTG
ACGGCCCAAG	CGGCAGGTCC	TGCTTGTCGG	ATAAGTTGCT	TACTCAAAAA	GGTTGACGAC
1330	1340	1350	1360	1370	1380
CTGATCCGAA	ACTGGCAACA	GTGGCAGGAG	CAAAAGGCGC	AGCTGGGGAA	ATGCCAGGCT
GACTAGGCTT	TGACCGTTGT	CACCGTCCTC	GTTTTCCGCG	TCGACCCCTT	TACGGTCCGA
1390	1400	1410	1420	1430	1440
TGCGGAAAGC	TGAAAGCGGA	GGGAGGTTGC	GGCGGCGAAC	GGCAGAGCGA	AAACCTTTAA
ACGCCTTTTCG	ACTTTCGCCT	CCCTCCAACG	CCGCCGCTTG	CCGTCTCGCT	TTTGGAAT
				*	
1450	1460	1470	1480	1490	
CTGCTGGCTA	GCCGTGGAAG	CAACGAGCTA	ATGTGTAGTG	TATTGTGC	
GACGACCGAT	CGGCAGCTTC	GTTGCTCGAT	TACACATCAC	ATAACACG	

Table 13. Restriction endonucleases with one or two sites in the *kikB* region

Enzyme	# sites	Location, nucleotide from the Tn5 insertion in pCU51
<i>Asu</i> II	2	393 423
<i>Ban</i> II	1	853
<i>Bsp</i> 1286	1	853
<i>Bsp</i> MI	1	248
<i>Dra</i> I= <i>Aha</i> III	2	27 457
<i>Eae</i> I= <i>Cfr</i> I	2	843 1,024
<i>Fox</i> I	2	317 494
<i>Fsp</i> I= <i>Mst</i> I	1	1,064
<i>Hga</i> I	2	106 651
<i>Hinc</i> II= <i>Hind</i> II	1	869
<i>Hin</i> FI	2	744 1,191
<i>Hpa</i> I	1	869
<i>Mae</i> I	1	1,448
<i>Mst</i> I= <i>Fsp</i> I	1	1,064
<i>Nde</i> I	1	565
<i>Nhe</i> I	1	1,447
<i>Pvu</i> II	2	895 1,362
<i>Rsa</i> I	1	309
<i>Sac</i> II	1	843
<i>Xho</i> II	2	442 878

Table 14. Characteristics of the predicted ORFs present in the *kikB* region

ORFs present in the upper strand	Location	Molecular weight (Da)	# of amino acid
ORF125	212-589	14,194	125
ORF75	591-811	8,348	75
ORF51*	799-956	5,623	51
ORF100	1138-1440	11,723	100
ORF present in the lower strand towards <i>oriT</i>			
ORF60	412-230	6,848	60
ORF60*	282-100	7,432	60

Figure 27. Open reading frames detected in the pCU1 sequence present in the *kikB* region. The numbers indicate the coordinates (kb) of pCU1; some of the restriction sites mentioned in the text are shown. The dotted rectangle represents the location of *oriT*. The dark rectangle represents the *kikB* region which was expanded and shown below between coordinates 8.7 (nucleotide 1) and 10.2 (nucleotide 1488). The relevant potential ORFs on both strands are shown with an arrow indicating the orientation of transcription. Branched arrows indicate potential ORFs in the same frame. The lines below the ORFs represent the *kikB* region present in the derivatives pCU105 (ending at nucleotide 758 of the *kikB* region corresponding to coordinate 9.4 in the pCU1 map) and pCU109.

To gain insight into the possible functions of these ORFs, the predicted amino acid sequences were compared with the known sequences available in data banks using Blast and TFASTA. None of the ORFs described here showed extensive homology with other known proteins so that this approach did not provide clues to their function.

Analysis of pCU1::Tn5 deletion derivatives pCU101, pCU105 and pCU109 revealed that only pCU109 expressed a Kik⁺ phenotype; pCU101 and pCU105 had a Kik⁻ or Kik⁺⁻ phenotype. This result implicates the region present in pCU109 and absent in pCU101 and pCU105 as being responsible for the KikB phenotype. The region mentioned above could be located between nucleotide 940 (map location of the Tn5 insertion in pCU105) and the end of the region at coordinate 10.2 in the pCU1 map. This region excludes the ORFs located between coordinates 8.7 to 9.4 in the pCU1 map, leaving only ORF100 as a product that may be necessary for the KikB phenotype (see Figure 26). However, and as pCU56 (coordinates 5.7 to 11.2 in the pCU1 map) was shown to be not lethal to the *Klebsiella* or to itself in this host, ORF100 must be insufficient for the phenotype. One or more of the other regions present in pCU101 is also needed. Further studies will be needed to identify these other region(s) and to understand the mechanism by which they confer plasmid stability in *Klebsiella*. However, their immune phenotype seems to be only a secondary consequence of ensuring this stability.

Further Discussion

There is now overwhelming evidence that when any naturally occurring plasmid is studied in some detail, this reveals that it has evolved a variety of mechanisms to ensure its stability in bacterial populations. Some of these mechanisms such as the ability of conjugative plasmids to be transmitted "horizontally" through the cells in a bacterial population or the ability of some plasmids to produce antibiotic proteins (bacteriocins) that act selectively on other members of the same or closely-related species have been known for a long time. Others, most of which seem to operate from within the cell have only recently come to light. The better-studied of such systems, most of which have been studied in *E. coli* K12, were reviewed (Literature Review). For plasmids or plasmid groups that are known to have a broad host range as in the case of pCU1 (Iyer, 1989), mechanisms have also to be evolved to ensure that the stability systems operate and are controlled in more than one host species into which the plasmid can gain entry (most often by bacterial conjugation).

Several of the systems described in the Literature Review were identified by discovering that clones carrying a particular region of a naturally occurring plasmid could not be selected following a plasmid transformation reaction in *E. coli* unless the clone itself or the recipient being transformed carried one or more other regions. The most general term used for these two sets of regions are *kil* and *kor* with letters to indicate cognate pairs showing

specificity to one another (e.g. *kilA* and *korA*). In contrast, the Kik^+ phenotype and immunity to it were discovered by the observation that when auxotrophic *E. coli* carrying pCU1 (or any other conjugative N group plasmid) was mated with prototrophic *Klebsiella* and the viability of all potential recipients (not transconjugants only) scored, there was 90-99% reduction in their viability. This was not observed in similar matings with *E. coli* recipients. Thus there is an operational distinction between the two phenotypes and the Kik^+ phenotype is apparently host-specific. A further feature of the *Klebsiella* system that motivated this study was the preliminary observation that *Klebsiella* that survived (1-10%) and carried the plasmid were not only themselves immune to the Kik^+ phenotype when mated with *E. coli* carrying pCU1 but derivatives of the *Klebsiella* that had lost the plasmid or plasmid DNA were also immune. This latter observation is unique and seemed to imply that plasmid pCU1 when introduced into *Klebsiella* was acting as a mutator of the *Klebsiella* chromosome. The objective of this study was to identify and characterize the region or regions on plasmid pCU1 that was needed for this Imm^+ phenotype to arise, as this in turn might provide clues to the nature of the mutation in *Klebsiella*. A region c. 1.39 kb of the 39 kb plasmid has been defined and characterized as being both necessary and sufficient for the Imm^+ phenotype to arise. In the attempt to define this region, a region that is conditionally lethal to

both *Klebsiella* and to *E. coli* was discovered. This region is called *kikC'* or *kilC'* (depending on whether the host is *Klebsiella* or *E. coli*) and in this discussion, it will be shown that one and the same gene is probably responsible for lethality in both hosts. In *E. coli* a second gene called *korC'* can override the lethality of *kikC(kilC)* as is the case for several other *kil/kor* type systems found in plasmids such as pKM101, RK2 in *E. coli* and pIJ101 in *Streptomyces lividans* where the presence of one or two *kor* regions can neutralize the effect of a *kil* region. In *Klebsiella* however, while the same *kor* gene is necessary to override the lethality of *kikC*, it is insufficient and the *Klebsiella* must in addition be mutated to the immune state. The rest of this discussion will be speculative, focussed on the different aspects of the phenomena and the questions they still raise.

Nucleotide sequence structure of the *kikA-kikC-korC-imm* region

It can be seen from Figure 22, that the region that is now defined as *kikC* is distinct from the region previously defined as *kikA*. It may be recalled that in the presence of functional *kikA* and in a mating reaction where a *KikA'* plasmid is transmitted from *E. coli* to *Klebsiella*, the viability of the recipients drop. In this laboratory (Holčík, pers. commun) it has been found that the reading frames shown as ORF104 and ORF70 in Figure 15 are needed for this

phenotype. It may also be noted that this KikA-determined phenotype and the Imm⁺ phenotype are observed with the parental plasmid pCU1 or the clone pCU109 both of which contain *kikC* and *korC*. Transposon Tn5 derivatives such as pCU66 or deletion derivatives like pCU403 (Figure 6) that do not display the Kik⁺ phenotype still display immunity to pCU109. The region determining immunity is therefore separable from *kikA*.

In Figure 22, the region shown as *kikC*, contains putative promoter sequences before ORF63 and also before ORF101. Thus, and in theory, ORF63, ORF123 and ORF101 proteins could be produced from this region. Furthermore, there is a one-base overlap between a stop and a start codon at ORF63-ORF123 junction raising the possibility of frame-shifting and the formation of a large "ORF63-ORF123" protein where the ORF123 portion will have an altered amino acid sequence. I have shown that inactivation of the promoter before ORF63 destroys *kikC* function indicating that this is the relevant promoter for *kikC*. A mutation that destroys *kikC* activity is a base substitution in the second codon of ORF123, a position where there is also a putative divergent promoter for ORF101 and ORF234. As it has been surmised that the functional promoter for *kikC* is not this promoter, the loss of the Kik⁺ phenotype in this mutant is likely to be due to the inactivation of ORF123 (or "ORF63-ORF123"). In addition to this mutation, there are two other mutations

that destroy *kikC* activity. Neither of these mutations affect the sequence of ORF234 or its promoter. The *kikC* phenotype is therefore not caused by ORF234.

Relationship between *kikC*, *korC* and immunity.

In pCU1, the conjugative *tra* region as defined by cloning and transposon mutagenesis in *E. coli* begins at about coordinate 32.0 to 32.4. *kikC* is therefore close to but outside this *tra* region. In the related plasmid pKM101 which has the same *tra* system as pCU1, a non-clonable region called *kilA* was located between two *kor* genes called *korA* and *korB* both of which were necessary to neutralize lethality conferred by *kilA* (Winans and Walker, 1985a). In this region, the sequence of pKM101 is identical to that of pCU1 and *kilA* of pKM101 is now believed to be part of an extended region of a *tra* operon. Accordingly, *kilA* of pKM101 has been renamed *traL* (Winans, per. commun.). In pCU1 the analogous sequence is ORF234 (Figures 22, 14) and its striking similarity to *virB1* of the Ti plasmid of *Agrobacterium* was emphasized. The pCU1 region (ORF63-ORF123) that is defined as *kikC(kilC)* (Figure 22) is different from this gene and reading in a direction opposite to it. In the *korC* region as shown in Figure 22, ORF93 is the *korC* gene based on the effect of deleting sequences from its promoter end that was described in Results, Part I.

While a plasmid carrying the *korC* region alone is able to protect *E. coli* from the lethal effect of *kikC(kilC)*, it is unable to do so in *Klebsiella*. In this species, a second requirement is that the host chromosome undergo a mutation that was first detected by its ability to confer immunity to the Kik⁺ phenotype. However, immunity by itself is not sufficient to protect this host against the lethality of *kikC*. *korC* is needed in the immune *Klebsiella* as well.

Neither *korC* nor *kikC* by themselves confer immunity. Most of the plasmid derivatives that gave rise to immune *Klebsiella* carried the entire *kikC* to *korC* region including the intervening sequences. A *Klebsiella* carrying *kikC* and *korC* regions without the intervening sequences could not be constructed (unless the *Klebsiella* has been previously rendered immune). However the role of *korC* may simply be to provide protection against the lethality of *kikC* and it may not be necessary for immunity to arise if *kikC* is inactivated. This focuses attention on the *virB1*-like ORF234 sequence as being essential for immunity to arise. So far, the similarity of its sequence to the *virB1* gene of the *vir* regulon of Ti plasmids has led to the notion that both may be needed for the plasmid transfer to other bacteria or to the plant nucleus respectively. The evidence for this is circumstantial and *virB1* mutants unlike other *virB* mutants attenuate but do not eliminate virulence. Further and detailed studies are needed on both *virB1* and the *virB1*-like gene of pCU1.

Can immune *Klebsiella* arise by selection of a pre-existing sub-population?

A relatively simple hypothesis that was considered to explain the emergence of immune *Klebsiella* is that even though populations are initiated from single cells, they may be heterogenous and contain a proportion of Imm⁺ cells. Since the plasmid with the active *kikC* gene can exert a strong selective force, this could serve to simply select clones of the rare Imm⁺ *Klebsiella*. To determine if Imm⁺ *Klebsiella* preexist in the population before exposure to pCU1, Gill (1985) screened populations started from 2600 individual colonies and found all of them to be susceptible to Kik⁺ phenotype. In the present study, I have generated two derivatives (pAAG4/pAAG445 and pAAG3/pAAG345) and shown by sequencing that they have deletions of 67 bp (pAAG4) and 274 bp (pAAG3) from the C terminal end of *kikC*. These derivatives are transmitted to Imm⁺ *Klebsiella* either by conjugation or by transformation with a high frequency. Nevertheless, both derivatives give rise to Imm⁺ *Klebsiella*. This observation does not support a selection hypothesis and since immunity persists when these plasmids are cured, it indicates that these plasmids can exert a mutator effect in the absence of functional *kikC*. These observations also indicate that the *virB1*-like sequence ORF234 that is present in these derivatives with *kor* did not exert a lethal effect.

Some important major questions require further comment although I am unable to provide answers to them at the present time.

(1) Why is *kor* sufficient to counteract *kil* in *E. coli* but not in *Klebsiella*? We do not know how *kor* acts on *kil* in either *E. coli* or immune *Klebsiella*.

However, the evidence does suggest that one and the same *kor(korC)* region is sufficient in both of these hosts to act on the same lethal gene (*kikC*). However, in non-immune *Klebsiella* it is insufficient. This directs attention to differences between *E. coli* and *Klebsiella* with respect to either expression signals or accessory factors either of which can be altered in *Klebsiella* probably by a single chromosomal mutation. For example, survival may be determined by the ratio of the production of these two proteins in the two hosts or their respective half lives. A systematic examination of this question is therefore possible before which methods of detecting and quantitating these proteins need to be developed.

(2) What could the nature of the mutation in immune *Klebsiella* be? Attention is drawn to the fact that all previously known mechanisms of overcoming the effect of a potentially lethal protein (Reviewed in "Literature Review") are inadequate to explain the unique phenomenon described here as it was demonstrated that cells were immune even when the plasmid had been spontaneously cured. A possibility that was then considered was that a portion

of the plasmid inserted into the chromosome mutating it in much the same manner as a site or region-specific insertion sequence element. If such an event occurred, it would have to involve an element much less than 400 bp as under hybridization conditions where a strong signal was given by a 400 bp artificial insert none was observed in immune and plasmid-cured *Klebsiella*, when pCU1-labelled plasmid DNA was used as a probe.

The mutation induced in *Klebsiella* is likely to be specific as immune *Klebsiella* have generally been found to be prototrophic. These observations suggest that the mutation is site-specific and probably caused by a recombination-type event such as site-specific recombination between direct repeats in the *Klebsiella* chromosome. A potential recombinogenic protein may be already present in *Klebsiella* being activated in the presence of the plasmid region. After the recombination event has occurred it would not then matter if the plasmid is lost from the cell. Alternatively, the plasmid region itself may specify the recombinogenic protein. This latter possibility seems unlikely as none of the polypeptides in this region from *kikC* to *korC* have features consistent with this type of protein. Since (at least in the absence of selection for this event) immune *Klebsiella* have not been found to revert to a non-immune state, the nature of the mutation may be a deletion in a region of the *Klebsiella* chromosome. Such a region now needs to be identified and

characterized. When this is done, it will help understand the nature of immunity and also why in such mutated cells, but not in unmutated cells, *korC* is sufficient to neutralize the effect of *kikC*.

The second part of the results of this thesis was concerned with the role of the *kikB* region in the phenomenon of killing and immunity. The information available concerning the role of *kikB* was contradictory. It was considered as part of the *Kik*⁺ phenomenon because a plasmid containing the two regions *kikA* (in pCU101) and *kikB* (in pCU51) rearranged to be together in the same plasmid pCU114 (see Figure 25) restored the *Kik*⁺ phenotype that was absent in pCU101. The *kikB* region was also shown to be involved in the *Imm*⁺ phenotype (Gill, 1985) because transconjugants from pCU101 matings were *imm*⁺. The results presented in this work suggest that the role of the *kikB* region in killing and in immunity is only the consequence of its structural instability when introduced into the *Klebsiella* environment.

Analysis of *Klebsiella* transconjugants from pCU101 matings showed that they frequently undergo structural rearrangements; when wild type plasmids were isolated at a low frequency they were found to be able to give rise to *Imm*⁺ *Klebsiella*. The observation that pCU114 (Figure 25) killed *Klebsiella* efficiently (Thatte *et al.*, 1985b) agrees with the hypothesis that this region plays a role in the installation of the plasmid in the new host. An

excellent example of 'installation' genes are described in the leading region of the IncII plasmid Collb-P9. The region contains the *psiB* gene specifying a protein that inhibits induction of the SOS response of the cell (Golub *et al.*, 1988) and the *ssb* gene specifying a single stranded DNA-binding protein which might participate in the metabolism of the plasmid during bacterial conjugation (Howland *et al.*, 1989). These two products are expressed upon zygotic induction following conjugal transfer (Jones *et al.*, 1992). The leading region of Collb-P9 also encodes an antirestriction system (the *ardA* locus) which alleviates restriction of DNA by all three families of type I restriction enzyme (Delver *et al.*, 1991; Read *et al.*, 1992)) and a *hok/sok* like system that increases plasmid stability by killing plasmid free segregants.

These observations suggest that detailed analysis of the leading region of pCU1 may help explain the basis of the host-specific structural instability of derivatives lacking this region. Furthermore, it was shown that this region that contributes to structural stability in *Klebsiella* could be dispensed with in the mutated immune derivatives of the same *Klebsiella*. Together with the experiments in the first part of "Results and Discussion", this suggests that the physiological effect of the mutation in *Klebsiella* is not directed to a specific lethal gene but that it can operate to counteract the lethality (to the plasmid or

its host) of several potential killing systems. The existence of such a system could contribute to the expansion of plasmid host-range.

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