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Molecular Cloning, Characterization, and
Nucleotide Sequence Analysis of *kikA*,
a Region of the IncN Plasmid pCU1
Required for Killing of
Klebsiella oxytoca

by

Paul N. Hengen, B.S., M.S.

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

Department of Biology
Carleton University
Ottawa, Ontario

April, 1991

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**"Molecular Cloning, Characterization, and
Nucleotide Sequence Analysis of *kikA*,
a Region of the IncN Plasmid pCU1
Required for Killing of**

***Klebsiella oxytoca*"**

submitted by

Paul N. Hengen

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

Carleton University - April 1991

Chairman, Department of Biology

Supervisor

External Examiner

Abstract

Broad host range plasmids of incompatibility group N and to some extent those of group P and W have the ability to kill *Klebsiella oxytoca* recipients upon bacterial conjugation. Previous studies of the IncN plasmid pCU1 have indicated that two regions, *kikA* and *kikB*, are involved in this phenomenon (Thatte *et al.*, 1985a). In this study, the *kikA* region of the IncN plasmid pCU1 was analyzed by molecular cloning, deletion, and nucleotide sequencing.

Deletion analysis localized the *kikA* region to within a 0.5 kb DNA fragment. When this fragment was mobilized into *K. oxytoca* by *Kik*⁻ helper plasmids, the recipients were killed. Further analysis of this fragment by nucleotide sequencing revealed the possible involvement of two open reading frames, ORF104 and ORF70. Two transposon Tn5 insertions that were phenotypically *Kik*⁻ were localized to the *kikA* region by nucleotide sequencing confirming that this region is responsible for killing. These insertions disrupted ORF104 but not ORF70. A possible role for each was considered and based upon the properties of the putative polypeptide product from ORF104, we predict that ORF104 may be associated with the cytoplasmic membrane.

Hybridization studies using the *kikA* fragment as probe provided molecular evidence that *kikA* is highly conserved among those plasmids of incompatibility group N. This suggests that there may be an advantage for retaining this function.

However, plasmids of incompatibility groups P and W, which are also known to produce a low level of killing, did not hybridize to the *kikA* probe.

A cloned DNA fragment containing the *kikA* region was used in a series of genetic experiments to investigate the possible involvement of the pCU1 origin of transfer replication (*oriT*). When *kikA* was mobilized by the IncP transfer system, killing was observed at a level comparable to that of pCU1. In addition, a role of the conjugative process itself in killing was ruled out by placing *kikA* under the transcriptional control of the inducible *tac* promoter. Upon induction with IPTG, killing of *K. oxytoca*, but not of *E. coli* was observed. We conclude from this that the *kikA* product is responsible for specifically killing *K. oxytoca* and that killing can be mediated independent of the mating event.

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List of Abbreviations

ATP	Adenosine triphosphate
Ap	ampicillin
bp	base pair(s)
°C	degrees centigrade
Cm	chloramphenicol
cfu	colony forming units
Ci	Curie(s)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DCDS	donor cell DNA synthesis
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram(s)
hr	hour(s)
Inc	incompatibility
IPTG	isopropyl-β-D-thiogalactopyranoside

<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>
<i>kor</i>	gene(s) required to override the effect of <i>kil</i> gene(s)
Km	kanamycin
kb	kilobase(s)
<i>lacZ</i>	gene encoding the enzyme β -galactosidase
LB	Luria broth
mg	milligram(s)
ml	milliliter(s)
M	molar (moles per liter)
mM	millimolar (10^{-3} moles per liter)
min	minute(s)
Nal	nalidixic acid
ng	nanogram(s)
nt	not tested
ORF	open reading frame
<i>oriT</i>	origin of transfer replication
PEG	polyethylene glycol
P_{tac}	artificial hybrid promoter
r	resistance

RCDS	recipient cell DNA synthesis
RF	replicative form
RNA	ribonucleic acid
Rif	rifampicin
rpm	revolutions per minutes
sec	second(s)
s	sensitivity
ss	single stranded
SDS	sodium dodecyl sulfate
Sp	spectinomycin
Sm	streptomycin
Su	sulfonilamide
Tc	tetracycline
TE	Tris-EDTA buffer
Tn	transposon
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
<i>tra</i>	conjugative transfer gene(s)
U	unit(s)
μ Ci	microcurie(s)
μ g	microgram(s)
μ l	microliter(s)

UV	ultraviolet
V	volt(s)
v/v	volume per volume
VCC	viable cell count
w/v	wieght per volume
xg	multiplicity of acceleration due to gravity
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
2xYT	two-fold concentration of yeast tryptone media
%	percent
+	positive phenotype
-	negative phenotype
<	less than
>	greater than

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INTRODUCTION

Plasmid pCU1 is a conjugative R plasmid of the incompatibility (Inc) group N. Plasmids of this group are known to be transmissible to and maintained in a wide variety of Gram-negative bacteria including the Enterobacteriaceae. Plasmid pCU1 is therefore considered to be a broad host range plasmid. Figure 1 shows a physical and genetic map of pCU1.

Plasmids of N, P, and W incompatibility groups have common mechanisms that give them broad host range capability. These broad host range plasmids therefore appear to have conserved these properties. The killing phenomenon, designated phenotypically as Kik⁺, is also a common property of N, P, and W Inc group plasmids (Rodriguez and Iyer, 1981). In addition, *Escherichia coli* carrying a plasmid of these types are sensitive to the icosohedral phage PRD1 (Olsen *et al.*, 1974). Susceptibility to PRD1 is related to the Kik phenomenon since transposon Tn5 mutations within the *kikA* region not only abolish the Kik⁺ phenotype, but also render the cell PRD1 resistant (Thatte *et al.*, 1985a).

The killing of *Klebsiella* (Kik) is a phenomenon by which recipient cells of *Klebsiella oxytoca* lose up to 95% of viable cell count whereas recipient cells of *E. coli* or even the very closely related *Enterobacter aerogenes* do not. The Kik phenomenon was first described using *Klebsiella pneumoniae* M5a1 as the tester strain in 1981 (Rodriguez and Iyer, 1981); However in 1977, *K. pneumoniae* M5a1 had been reclassified as a separate and distinct species named *K. oxytoca*. The

Figure 1. A circular physical and genetic map of the IncN plasmid pCU1. The restriction enzyme map positions are based on those of Konarska-Kozłowska *et al.* (1983) with some modifications. The numbers correspond to the kb coordinates of plasmid pCU1. Restriction enzyme sites are shown outside the circle. The functional complementation groups denoted by letters within the transfer region are based on those of Thatte *et al.* (1985a). The position of *oriT* is based on mapping and sequence analysis (S. Paterson, M.Sc. thesis, Carleton University, in preparation). Mapping of some restriction enzyme sites is based on sequence data of V. Thatte (personal communication). Rep is the replication region. Sp^r, Sm^r, and Ap^r are regions conferring resistance to antibiotics spectinomycin, streptomycin, and ampicillin respectively. *kikA* and *kikB* are regions involved in killing of *K. oxytoca* (Thatte *et al.*, 1985b).

following is a synopsis of the history of *K. pneumoniae* M5a1.

The strain M5a1 was first isolated in the 1930's by Dr. E. McCoy at the University of Wisconsin. At this time, it was classified as *Aerobacter aerogenes*. In 1965 the strain was reclassified as *K. pneumoniae*. M5a1 was again reclassified as *K. oxytoca* in 1977 (Yocum, 1989). *K. oxytoca* is distinguished from *K. pneumoniae* by its ability to produce indole from tryptophan. *K. pneumoniae* does not produce indole. More recent studies show that *K. oxytoca* has less than 70% DNA homology with the *K. pneumoniae* typestrain (Bergan, 1984). In 1981, M5a1 was renamed UN by the research group at the University of Wisconsin (MacNeil *et al.*, 1981). The tester strain *K. oxytoca* UN2979Rif^r used in this study is a LacZ⁻ His⁻ rifampicin resistant derivative of UN. Throughout this thesis, I will use the taxonomy as described in the Bergey's Manual of Systematic Bacteriology (Brenner, 1984). It should be noted that earlier studies conducted with the killing of *K. pneumoniae* M5a1 are describing the same phenomenon.

Rodriguez and Iyer (1981) have shown that the killing of *Klebsiella* requires mating. Transfer of pCU1 is much more efficient on solid media than in liquid media. When *E. coli* donors carrying pCU1 are mixed with *K. oxytoca* recipients in liquid media, the Kik⁺ phenotype is not observed. Bacterial matings performed by entrapping donors and recipients on nitrocellulose membrane filters are more efficient in DNA transfer and show the Kik⁺ phenotype.

Killing has been shown not to be mediated by any substance which diffuses from *E. coli* to *K. oxytoca* since cell-free culture filtrates did not produce the killing effect. Also, when a nitrocellulose filter is sandwiched between donors and recipients, killing is not observed. In addition, cell-to-cell contact is not sufficient to cause lethality since nalidixic acid, an inhibitor of DNA transfer between cells, also inhibits the Kik⁺ phenotype (Gill and Iyer, 1982). Although these reports have excluded the role of a bacteriocin-like substance that diffuses through the media or is cell-bound, it is still quite possible that the mechanism of cell killing is similar to that of a bacteriocin. I have therefore included the current knowledge of bacteriocin gene analysis within the literature review section of this thesis. The mechanism of killing in *Klebsiella* does appear to be mediated from within the cell and is therefore different than any bacteriocin studied.

The transfer (*tra*) functions of pCU1 are contained within a single continuous segment of the plasmid. The *tra* region is composed of eight distinct complementation groups (A through H). Six of these are concerned with the production of N-type pili (see Figure 1; Thatte *et al.*, 1985a). Since killing is mediated by conjugation, the possibility that part of the *tra* region of pCU1 was involved in the killing mechanism was considered. By using Tn5 generated mutations within each of the transfer complementation groups, Rotheim *et al.* (1988) have demonstrated that each of the individual components of the transfer region does not

specify any product involved in the Kik⁺ phenotype. This study however, has not excluded a role of bacterial conjugation in the killing phenomenon. I have therefore included a review of the conjugative process of the classical plasmid F. Two phenomena associated with transfer of F plasmid are zygotic induction and lethal zygosis. Both of these result in a loss of recipient cell viability following mating. I have therefore included a review of these as well.

Genetic analysis of pCU1 by means of transposon and deletion mutagenesis has provided evidence that two separate regions of the plasmid, besides the transfer region, are required to kill *K. oxytoca* upon conjugative transfer (Thatte *et al.*, 1985b). Figure 2 shows a map of pCU1 summarizing the data that lead to this conclusion. One region designated as *kikA* is located next to the left end of *tra* on the pCU1 map. This was determined by transposon Tn5 insertions into three different positions within pCU1. Those three insertions were closely clustered between pCU1 coordinates 30.5 and 31.5 kb. Each of these derivatives was found to be Tra⁺ Kik⁻, whereas pCU1 was found to be Tra⁺ Kik⁺.

A second region designated as *kikB* was found to be located at the end of *tra* opposite *kikA*. This was determined by cloning the *tra* region of pCU1 into the plasmid vector pACYC184 (Chang and Cohen, 1978; Thatte and Iyer, 1983). Recombinant plasmids, pCU101 and pCU105, contain the entire *tra* region (including *oriT*, a region required *in cis* for plasmid mobilization) and *kikA*. pCU101

Figure 2. A pCU1 map organized according to functional domains. Two intergenic regions span from 9.0 to 17.0 and from 26.0 to 30.0 on pCU1. *kikA* and *kikB* are two regions previously identified as responsible for the killing of *K. oxytoca*. *kikA* was identified by Tn5 insertions indicated by arrows. The region of pCU1 between 7.0 and 16.0 has been expanded to show the *kikB* region. Plasmid constructs defining *kikB* are shown in the expanded area. Shaded areas correspond to DNA from pCU1 cloned onto pACYC184 for the construction of pCU101, pCU105, and pCU109. The leftward limit of *kikB* is defined by the map position of Tn5 insertion 88 within pCU1 that was used for the construction of plasmid pCU109 (Thatte *et al.*, 1985a). The rightward limit of *kikB* is defined by the nucleotide sequence of pCU101 (V. Thatte, personal communication).

and pCU105 show a reduced killing of *Klebsiella* compared to a similar construct, pCU109 containing the *kikB* region. The *kikB* region was mapped to a 1.8 kb region at the end of *tra* near *oriT* but is not included within the transfer region. The possibility was left open that *kikB* may only be a very small part of this region because 33 Tn5 insertions in the vicinity of *kikB* did not affect the Kik⁺ phenotype (Thatte *et al.*, 1985a). To date, no mutations within the *kikB* region have been isolated. The reason for an apparent increase in killing when *kikB* is present is unclear.

The *kikA* and *kikB* regions have previously been physically separated on different plasmids. Evidence was presented that both must be mobilized into *K. oxytoca* simultaneously for killing to occur (Thatte *et al.*, 1985b). Killing did not occur if either *kikA* or *kikB* is first established in *Klebsiella* on a plasmid and the second region introduced subsequently on a second plasmid (Gill, 1985). Thus, it appears that *kikA* and *kikB* act in a coordinated manner during conjugation. *K. oxytoca* survivors of plasmid pCU1 transfer carry the antibiotic resistance markers of pCU1 and are able to kill other *Klebsiella* recipients during conjugation (Thatte *et al.*, 1985b). This suggests that killing is also associated with transient expression of genes specifying the killing functions. In addition, when pCU1 is transferred by mating from *E. coli* or *K. oxytoca* to *E. coli* recipients, or from *E. coli* donors to *E. aerogenes* instead of *K. oxytoca*, recipient cells are not killed. The loss of viability is

thus a host cell-specific phenomenon.

Since transposon mediated mutations within *kikA* have been produced and it has been shown that the mutations can be rescued *in trans* within *K. oxytoca* (ie. mutations in *kikA* are recessive), it was surmised that *kikA* encodes an intracellularly diffusible product within *K. oxytoca*. The working hypothesis of this thesis is that, in *K. oxytoca*, the activity of a product specified by *kikA* causes lethality. This thesis is directed at the molecular study of the *kikA* region and the relationship of *kikA* among those conjugatively self-transmissible plasmids of broad host range capability. An understanding of the mechanism of *kik* "gene" control may provide valuable insights for a better understanding of coordinated or cell specific gene regulation as a whole. At the present time, the molecular mechanism by which the killing phenomenon occurs is unknown.

Comparison of pCU1 and pKM101 genetic and physical maps

The IncN plasmids pCU1 and pKM101 have recently been the subject of molecular study (reviewed by Iyer, 1989). The plasmids have different origins. pCU1 (39 kb) was originally isolated from a *Salmonella* strain in Eastern Canada (Khatoon and Iyer, 1971). pKM101 is a 35.4 kb deletion derivative of R46, an R plasmid isolated from a *Salmonella* strain in the United Kingdom (Drabble and Stocker, 1968). Despite their diverse backgrounds, pCU1 and pKM101 show striking similarities in both their physical and genetic organization. Figure 3 shows the genetic, functional and complementation map of the transfer regions of each plasmid. It is apparent from the location of restriction enzyme sites within this region that the two are very closely related and may have only recently diverged. The *tra* region of pKM101 was found to consist of 11 genes whereas within the *tra* region of pCU1, only 8 complementation groups have been identified (Thatte *et al.*, 1985a). The complementation groups are presumed to be single transcriptional units. Both *tra* regions may be disrupted by two non-transfer segments thus creating three subdivisions. Tn5 mutations within the *tra* region of pCU1 can be complemented by pKM101, providing evidence that the transfer regions may be functioning by an identical mechanism (V. Thatte, personal communication). Like pCU1, pKM101 has the ability to kill *K. oxytoca* (Kik⁺) (Rodriguez and Iyer, 1981; Winans and Walker, 1985a). However, the regions necessary for the Kik phenotype of pKM101 have

Figure 3. A comparison of plasmids pCU1 and pKM101. The transfer regions of each of these near identical IncN plasmids are shown side by side. The complementation groups *traA* through *traH* are shown for pCU1 and *traA* through *traK* are shown for pKM101. The functional map of pKM101 is based on that of Winans and Walker (1985a). The functional map of pCU1 is based on that of Thatte *et al.* (1985a). *kikA* and *kikB* regions are shown as shaded areas on pCU1. No *kik* regions have been mapped on pKM101. *oriT* is a region required *in cis* for plasmid transfer. Restriction enzyme sites shared between the two plasmids are indicated.

not been mapped

It is not known why two non-transfer segments exist within the *tra* region of both pCU1 and pKM101. On pKM101, these interrupting segments code for: 1) entry exclusion (*eex*) (Winans and Walker, 1985b), 2) a periplasmic endonuclease (*nuc*), and 3) fertility inhibition of IncP plasmids (*fip*) (Winans and Walker, 1985d). A more detailed comparison of the transfer regions of pCU1 and pKM101 is shown in Figure 4. On pCU1, these relative positions are of unknown function at present; however, no Tn5 insertions which abolish the Tra⁺ phenotype were mapped at these locations. It is possible that these regions exclude transposon mutagenesis due to concurrent instability of the plasmid.

The functional organization of pKM101 has been described using deletion and transposon insertion analyses (Langer *et al.*, 1981). Two loci, *kilA* and *kilB*, on pKM101 have been identified which have the capacity to cause lethality within *E. coli*. In addition to these, pKM101 has corresponding *kor* functions which are necessary to counteract the effects of the *kil* genes. *kilA* is located at the end of the transfer region near *traA*. *kilB* is located within complementation group E of the transfer region. In the presence of *korB*, expression of *kilB* is involved in conjugal transfer of pKM101. *kilA*, however, is not involved in transfer but is responsible for abnormally slow growing colonies on minimal glucose media when pKM101 is present in the cell. This has been termed the Slo⁺ phenotype. Mutations in *kilA*

Figure 4. A detailed comparison of nearly identical plasmids pCU1 and pKM101. Regions of pKM101 not involved in conjugative transfer are described in the text.

kilA = region lethal to *E. coli* cells

korA = region required to suppress killing by *kilA*

kilB = region lethal to *E. coli* cells

korB = region required to suppress killing by *kilB*

eex = region specifying entry exclusion

nuc = region coding for a periplasmic endonuclease

fip = region specifying fertility inhibition of IncP plasmids

stb = region required for plasmid stability

mucA/mucB = mutagenesis enhancement

slo = region responsible for slow growth of *E. coli* on glucose minimal agar

REP = replication region

therefore have a *Slo*⁻ phenotype and grow quite normally (Winans and Walker, 1985c).

Of particular interest concerning the *Kik*⁺ phenotype is the area of pKM101 which corresponds to the region on pCU1 designated as *kikA*. Within pKM101, this region was found to contain a gene which is potentially lethal to an *E. coli* host, *kilA*, and two override genes, *korA* and *korB*. Evidence that *kilA* is able to kill and/or regulate cell growth of *E. coli* cells, and its location with respect to the transfer region suggests that it may be involved in killing of *K. oxytoca*.

The region on pKM101 corresponding to *kikB* of pCU1 is uncharacterized; however, it has been observed that at least one and possibly two *kil* genes reside close to the *mucAB* genes of pKM101, just 3 to 5 kb (22.5 to 28 kb on the pKM101 map, Figure 4) away from the end of *tra*. Based on the genetic map of pCU1, it is possible that the uncharacterized region of pKM101 between *oriT* and *mucAB* contains a gene or genes required for the *Kik*⁺ phenotype. One interesting aspect of this region is the mapping of a putative stability locus close to *oriT* on pKM101 (Winans and Walker, 1985a).

The map locations of *kil* and *kik* on pKM101 and pCU1 may suggest that the two genes are functionally analogous. If this is true, then perhaps the *kik* genes are similar to *kil* genes but are active or unchecked only in *K. oxytoca*. *E. coli* therefore contains inherent protection from *kik* but not from *kil*. However, Winans and

Walker (1985c) have shown that Tn5 insertions in *kilA* and *kilB* of pKM101 are Kik⁺ providing evidence that the *kil* genes of pKM101 are not involved in killing of *K. oxytoca*.

Plasmid stability and viability of host cells

Promiscuous plasmids are known to transfer to many different species of bacteria. The limiting factors for such promiscuity fall into two basic categories, the ability to transfer and/or receive the plasmid, and the ability to maintain the plasmid. When introduced to a new host, the plasmid encounters a completely different cytosolic environment. If the new environment is hostile, the plasmid may be degraded by endonucleases. Frequently, the plasmid is capable of transfer and is maintained for some time, but may be lost from the cell because it is either segregationally unstable or it has imposed a disadvantage onto the host relative to cells that do not carry the plasmid. In the case of instability, the plasmid may only appear in the cell transiently, being lost shortly after conjugation. When pCU1 or its minireplicon is conjugatively transferred to *Agrobacterium*, *Bradyrhizobium*, and *Rhizobium* species, the plasmid is only maintained for a short period of time in the absence of selection. However, the pCU1 minireplicon is stable within *E. aerogenes* (Krishnan and Iyer, 1988). This is an example of replication region dependency on host-encoded stabilization factors. Other plasmid-encoded stabilization factors account for the ability of broad host range plasmids to replicate in a variety of genetic backgrounds. Since pCU1 is able to kill *K. oxytoca* but not the very closely related *E. aerogenes*, mechanisms that stabilize the plasmid apart from that of the minimal replicon must be considered.

Recent studies have revealed that in addition to replication, plasmid stability is determined by a combination of 1) the ability to prevent multimerization or resolve multimeric forms of the plasmid, 2) active partitioning of the plasmid to daughter cells, and 3) the selective killing of cells that do not carry the plasmid (Iyer, 1990). In the following literature review, only the selective killing of cells is reviewed as it may be the one most directly related to the mechanism of killing of *K. oxytoca*. Studies of a number of different plasmids show that plasmid stability can be accomplished by a variety of methods, all of which penalize a host for not conserving the plasmid.

LITERATURE REVIEW

Section I. Plasmid associated functions affecting viability of host cells

At present, three types of killing genes are known to be associated with plasmid stability in Gram-negative bacteria: 1) those that prevent cell division by a mechanism coupling cell proliferation to plasmid inheritance such as *ccdA/ccdB* of F, 2) those that kill the cells not containing the plasmid by a mechanism that disrupts the cellular membrane such as *hok/sok* and *hok*-related genes, and 3) those widely dispersed genes conferring stability under a regulatory network that prohibit separation of particular plasmid regions such as the *kil/kor* regulon of RK2.

Coupled cell division genes

ccdA/ccdB system of F plasmid

The F plasmid has been shown to carry a region of DNA that allows the plasmid to be stably maintained without selective pressure. This region has been termed *ccd* for coupled cell division. *ccd* lies outside of the DNA region required for replication of the plasmid DNA and is also apart from the region required for plasmid partitioning, *sopA*, *sopB*, and *sopC* (Ogura and Hiraga, 1983a). The *ccd* region of 0.76 kb contains two distinct functional domains, *ccdA* and *ccdB*. The concerted effort of the *ccdA/ccdB* genes ensure that each cell contains at least a single copy of F (Donachie and Robinson, 1987). Ogura and Hiraga (1983b) have shown that *ccdB* specifies an inhibitory function while *ccdA* suppresses the

inhibition. Hiraga *et al.* (1986) have shown that the mechanism of killing by *ccdB* is internal to the cells and does not occur by cell-to-cell interaction since normal cells co-existing with plasmid-free segregants were unaffected. The effect of *ccdB* in the presence of an amber mutation within *ccdA* is to cause filamentation and death in the host cell, induction of prophage λ , and induction of *sfiA*, the chromosomal *E. coli* gene encoding a cell division inhibitor (Huisman *et al.*, 1980; Huisman *et al.*, 1984). Jaffé *et al.* (1985) suggested that all cells not receiving a copy of F are somehow destined to produce filaments and die.

Sequence analysis of *ccdA/ccdB* genes, also designated as *letA* (*ccdA*) and *letD* (*ccdB*) by Miki *et al.* (1984), has revealed two translational open reading frames (ORF) of 72 amino acids (*ccdA*) and 101 amino acids (*ccdB*). It has been proposed that the mechanism for the action of *ccdA/ccdB* is that *ccdA* is normally able to suppress the action of *ccdB* when a copy of the plasmid is present in the cell. If *ccdA* is lost from the cell, the function of the *ccdB* product can no longer be suppressed and cell division is temporarily inhibited by induction of *sfiA*. Presumably, cells escape the effect only after the *ccdB* product has been degraded, lost through dilution, or the daughter cell receives a copy of the plasmid. The expression of the *ccd* operon has been shown to be autoregulated (de Feyter *et al.*, 1989; Tam and Kline, 1989a) and that a complex of CcdA and CcdB proteins is responsible for repression (Tam and Kline, 1989b). This may suggest that the

mechanism for suppression could be an interaction between the *ccdA*-encoded protein and the *ccdB*-encoded protein. The molecular basis of *ccd*-mediated inhibition of cell division and suppression of *ccdB* activity is poorly understood.

kid/kis system of IncFII plasmid R1

Two separate regions of plasmid R1 are required for partitioning, *parA* and *parB*. In addition to these, a third region specifying stability, *parD*, has been identified (Bravo *et al.*, 1987). The stability provided by the *parD* region has been shown to be composed of two genes, *kid* (for *k*illing *d*eterminant) and *kis* (for *k*illing *s*uppressor). The *kid* gene product is a protein of approximately 12 kDa that kills cells not containing the suppressor function encoded by *kis*. Presumably, cells are killed when the plasmid is lost because the suppressor protein of approximately 9.3 kDa is less stable than the killing protein. The suppressor is believed to inhibit killing by becoming reversibly bound to or is somehow able to antagonize the killing protein. The mechanism by which cells are killed by the *kid* product is unknown, but comparison of the *kid* product to that of *ccdB* shows the mechanism is most likely different. Comparison of the suppressor proteins, however, has indicated that the antagonizing mechanism may be similar (Bravo *et al.*, 1988).

pemK/pemI system of IncFII plasmid R100

R100 is a 89.3 kb conjugative plasmid of incompatibility group FII closely related to R1. Two regions distinct from the replication region of R100 have been identified that enable the plasmid to be stably maintained in *E. coli*. A region designated as *stb* is located close to insertion element *IS1* (Miki *et al.*, 1980). The other, *pem* (for *p*lasmid *e*mergency *m*aintenance), is located far from *stb* and close but not within the replication region of R100 (Tsuchimoto *et al.*, 1988). The *pem* region consists of two genes, *pemK* and *pemI*. *pemK* encodes an 11.9 kDa (110 amino acids) polypeptide that inhibits cell division of plasmid-free segregants leading to death of these cells. *pemI* encodes a 9.3 kDa (85 amino acids) protein that inhibits *pemK* and preventing killing of the cell by *pemK*. The nucleotide sequence of the *pem* region shows that both *pemI* and *pemK* are most likely transcribed in that order from the same promoter located upstream of *pemI*. The sequence of the *pem* region is identical to the *parD* region (*kid/kis*) of plasmid R1 described above. The *pemK/pemI* region has no homology with and is therefore different from *parB* of R1 and from *ccdB* of F. It has been shown that *pemK* causes inhibition of cell division like that observed for *ccdB* of F, but that this is not due to the induction of the *E. coli* *sfiA* gene (Tsuchimoto and Ohtsubo, 1989). The mechanism by which the PemK protein inhibits cell division is unknown at present.

hok and *hok*-related genes*hok/sok* system of IncFII plasmid R1

R1 is an IncFII conjugative plasmid of 89.3 kb. Equal partitioning of plasmid R1 to daughter cells was shown to be lacking when a region designated as *par* was absent. Genetic analysis of the *par* region revealed two separate and independently operating stability functions, *parA* and *parB*. The combination of *parA* and *parB* results in complete stabilization of the plasmid in the absence of selective pressure (Gerdes, 1985). *parA* encodes a 320 amino acid protein shown to be involved primarily with partitioning of R1 (Gerdes and Molin, 1986). *parB* controls stability by a postsegregational killing mechanism. The *parB* locus codes for two genes, *hok* (for *host* killing) and *sok* (for *suppressor of killing*). Overexpression of *hok* causes rapid killing of cells by producing a highly toxic gene product while *sok* is able to counteract the killing effect of *hok* (Gerdes *et al.*, 1986). The *hok/sok* system of R1 ensures that the plasmid is not lost from the cell by killing those that do not contain the *sok* region. This is accomplished by non-suppression of *hok* mRNA remaining in the cell in absence of *sok* anti-sense RNA. Recently, Gerdes *et al.* (1990) have shown that the *sok* RNA decays at a rate much faster than that of *hok*. The killing of cells is thus due to translation of unprotected *hok* RNA left in plasmid free segregants. Interestingly, the *hok* RNA appears to be processed shortly after the decay of *sok* anti-sense RNA, giving rise to a shortened transcript (Gerdes *et al.*,

1990)

Genetic and sequence analysis of the *parB* region has indicated that a 52 amino acid hydrophobic protein of *hok* is responsible for killing (Rasmussen *et al.*, 1987). This protein is similar in properties to the product of an *E. coli* chromosomal gene *relF*, a membrane associated protein that when overexpressed, causes loss of cell membrane potential leading to cell death (Gerdes *et al.*, 1986). The *relF* gene may be directly related to essential maintenance functions of the cellular membrane, while *hok* may be a divergent *relF* that has been acquired by the plasmid. Currently, the mechanism by which *hok* and *hok*-related proteins cause a disruption of the cell membrane appears to be the same for all Hok-homologous proteins. However, the target for the activity of such an event is not known. The function of *relF* in *E. coli* is also not known (Bech *et al.*, 1985; Gerdes *et al.*, 1990).

Another *E. coli* chromosomal gene, *gef* (for gene expression fatal) has been isolated based on homology to *hok*. Recent evidence shows that a family of genes homologous to *gef* and *relF* are conserved in many different species of Gram-negative bacteria as well as on plasmids from a number of different incompatibility groups. The implication is that these genes may play an important physiological role (Poulsen *et al.*, 1989; Gerdes *et al.*, 1990).

flmA/flmB system of F plasmid

The F plasmid also contains a region homologous to *hok* that stabilizes the plasmid. This region has been independently identified and designated as *flmA/flmB* (for *F* leading-region *m*aintenance) by Loh *et al.* (1988) and as *stm* (for *s*tability *m*aintenance) by Golub and Panzer (1988). Sequence analysis shows that the region identified in each study is nearly identical to the coding region of *hok*, differing only by four bases in one position and an insertion of 21 bases in another. The predicted *stm* protein is 59 amino acids in length and approximately 6.9 kDa. Southern hybridization of this DNA showed no homology to plasmids not related to F including N3 (IncN) or RP4 (IncP) (Golub and Panzer, 1988). The mechanism of action of *flmA* and *flmB* of F appears to be the same as *hok/sok* of R1. *flmA* (*stm*) is the equivalent of *hok*, and *flmB* the equivalent of *sok*. *flmB* RNA suppresses the expression of *flmA* by binding to the transcript analogous to the anti-sense control exerted by the *sok* gene of R1 (Loh *et al.*, 1988).

The F plasmid contains a second region related to *hok* designated as *smB* (stable RNA *n*egative). Mutations within *smB* were isolated in the course of producing chromosomal mutations in *E. coli* affecting the degradation of stable RNA species (Ohnishi, 1975). The *smB* region was localized to a region between 1.4 and 2.5 kb on the F plasmid map (Ohnishi *et al.*, 1977) and subsequently cloned (Ohnishi *et al.*, 1981). The nucleotide sequence of *smB* (Akimoto *et al.*, 1986)

revealed a putative ORF that could encode a protein of 69 amino acids with significant similarity to that of *hok* (Gerdes, 1990). Akimoto et al. (1986) have suggested that expression of *smB* changes the transport and permeability properties of the cellular membrane since it increases the membrane permeability of *o*-nitrophenyl- β -D-galactoside. This would allow RNase I in the periplasm to pass through the damaged membrane accounting for the degradation of RNA within the cell.

Ohnishi and Akimoto (1980) have also shown that a number of IncA plasmids including R483 and the IncB plasmids R16 and R834 contain *hok*-homologous regions termed *pnd* (*promotion of nucleic acid degradation*). Nucleotide sequence analysis of *pnd* from R483 has shown that a putative ORF shares 54% homology to that of *smB*. Thus, *pnd* is considered a member of the *hok*-related protein family. This ORF encodes a polypeptide of 50 amino acids. Comparison of the hydropathic profiles of the *pnd*- and *smB*-encoded polypeptides showed a region of similarity that is strongly hydrophobic, a common property of membrane associated proteins. Interestingly, both *pnd* and *smB* contain leader sequences that may cause a loop structure believed to be involved in transcriptional regulation (Ono et al., 1987). The mechanism by which *smB* and *pnd* disrupt the cellular membrane is unknown.

kil/kor regulatory networks*kil/kor* systems of IncP plasmid RK2 and IncN plasmid pKM101

When the killing of *K. oxytoca* first appeared in the literature, the phenotype was described as Kil⁺. Another phenomenon that is operationally different has since been described as Kil. In this study, all references to the killing of *K. oxytoca* are described as Kik. References to the Kil phenotype are directed toward a phenomenon common among IncP and IncN plasmids in which particular regions of these plasmids (*kil*) are unable to be cloned without a corresponding override region (*kor*). In the study of another IncN plasmid pKM101, it was found that particular fragments of this plasmid could not be cloned. Localization of pKM101-encoded genes having the potential effect of causing lethality occurred when attempts were made to subclone a pKM101 restriction fragment near one end of the transfer region. Cloning of a region designated as *kilA* could only be accomplished if additional sequences of pKM101 were provided *in cis* or pKM101 itself was provided *in trans* (Winans and Walker, 1985c). The non-clonable regions were thus termed *kil* (*kill*) regions and their protecting regions termed *kor* (*kill-override*) to coincide with a similar system reported for plasmid RK2 (Figurski and Helinski, 1979). It is the *kil/kor* system of RK2 that has been the subject of intense study in the past and for which the most detailed information is available. A discussion of this system and its relevance to plasmid maintenance is therefore

pertinent.

In the broad host range plasmid RK2, *kil* genes were found by the inability to clone a 14 kb *EcoRI* fragment from pRK212.2, a derivative of plasmid RK2. Cloning of this fragment could only be accomplished if another fragment from the same plasmid was present. This is because the latter fragment suppresses the action of a lethal (*kil*) gene (Figurski and Helinski, 1979). Cloning of specific regions of RK2 has revealed that four such *kil* genes reside on the plasmid: *kilA*, *kilB*, *kilC*, and *kilD*. Each *kil* gene is normally counteracted by a specific *kor* gene or combination of *kor* genes. For example, *korA* is specific for prevention of lethality by *kilA*, and *korB* is specific for *kilB*. Detailed study of RK2 has shown that the interactions of *kil* and *kor* genes are very complex (reviewed by Thomas and Helinski, 1989). Figure 5 shows (A) a detailed functional map of RK2 and (B) the current knowledge of *kil/kor* regulatory systems of plasmid RK2 in schematic form. From a multitude of studies concerned with the *kil/kor* system of RK2, it is clear that these genes play an important role in controlling gene expression within RK2 at the level of transcription, particularly that of *trfA*, the gene responsible for initiation of DNA replication which acts at *oriV*, a site required *in cis* for replication. A full understanding of the regulatory circuitry of *kil* and *kor* genes in RK2 is still incomplete.

The *korA* and *korB* genes of RK2 are highly conserved within the IncP group

Figure 5. Genetic maps of the broad host range plasmid RK2.

A) circular map of RK2 showing the essential features for plasmid replication based on that of Thomas and Smith (1987). The *trfA* gene product is required for replication initiating at *oriV*. The (+) designates a positive effect on plasmid replication. *trfB* is required for stable maintenance by repressing the action of *kil* genes. *trfB* also represses transcription of *trfA*. The (-) designates a negative effect on replication. Plasmid regions specifying resistance to antibiotics ampicillin (Ap^r), tetracycline (Tc^r), and kanamycin (Km^r) are shown by dark lines. Transfer functions Tra1, Tra2, and Tra3 are also shown by thinner lines. *oriT* is a region required *in cis* for plasmid transfer. *oriV* is a region required *in cis* for plasmid replication. The arrow indicates the direction of replication beginning at *oriV*. *par* is a region located between coordinates 30.8 and 37.0 involved in stable maintenance and partitioning (Saurugger *et al.*, 1986).

B) linear map of RK2 showing the current knowledge of the *kil-kor* regulon. All regulatory interactions of *kil* and *kor* genes shown as lines have negative effects on gene expression. Replication of RK2 requires the interaction of the *trfA* gene product at *oriV*. The regulon is based on that of Thomas and Helinski (1989).

trfA = trans acting replication factor

trfB = trans acting inhibitor of *trfA*, *kilA*, *kilD* (*trfB*=*korA*=*korD*)

kil = regions lethal to *E. coli* cells

kor = regions required to suppress *kil* genes

kcr = regions under control of *korC*

kfr = regions under control of *korF*

inc = regions involved in expressing incompatibility

oriT = region required *in cis* for plasmid transfer

oriV = region required *in cis* for plasmid replication

Ap^r = ampicillin resistance

Tc^r = tetracycline resistance

Km^r = kanamycin resistance

plasmids. This suggests that there is an advantage to retain these regions. Figurski *et al.* (1982) have found that of fourteen naturally occurring IncP plasmids isolated from diverse bacterial species, all encode *kor* functions when tested in *E. coli*. Studies of the *kill/kor* functions of RK2 focused primarily on the premise that these regions are important in the maintenance of RK2 in a wide range of bacterial hosts (Bechhofer, 1984). In fact, it has been shown that *kil* and *kor* genes are involved in plasmid maintenance by interacting with essential replication determinants (Figurski, *et al.*, 1985). What is unclear at this point is how the *kill/kor* systems operate in various hosts for plasmid stability and whether they contribute to its broad host range capability.

kill/kor systems of *Streptomyces* plasmid pIJ101

pIJ101 is an 8.9 kb conjugative plasmid originally isolated from the sporulating Gram-positive bacterium *Streptomyces lividans*. It is considered to be a broad host range plasmid (Kieser *et al.*, 1982). Transfer of pIJ101 occurs at high frequency and has also been known to cause chromosome mobilization at a very high frequency. When mating is performed on solid media, the frequency of chromosomal transfer is high enough to produce lethal zygosis in the recipient cells. This killing effect can be seen as a spot or "pock" on a lawn of recipient cells growing on media which prevents the growth of donor cells. Any mutations that render the plasmid non-transferrable or cause instability of the plasmid in the donor

do not show this phenotype. A study of DNA insertions within pIJ101 has revealed that a region conferring plasmid stability lies outside the essential replication region. The stability was found to be due to the presence of two *kil/kor* loci; *kilA* and *kilB* are lethal to the cell unless the corresponding *korA* and *korB* are also present. *kilA* and *korA* appear to be close to or associated with one end of the transfer region while *kilB* and *korB* are separated by the transfer region. *korA* can override the effects of both *kilA* and *kilB*, but *korB* can only override *kilB* (Kendall and Cohen, 1987). Sequence analysis of pIJ101 shows that *kilA* is most likely a segment of a transfer protein of 621 amino acids or part of a mechanism controlling the transfer process. The *kilB* region shows a putative ORF of 148 codons that has a single predicted hydrophobic domain (Kendall and Cohen, 1988). Analysis of the promoter regions of pIJ101 shows that each corresponds to the consensus sequence utilized by *E. coli* RNA polymerase holoenzyme carrying the σ^{70} subunit (or *Streptomyces* σ^{35} subunit). Transcription of both *kilB* and *korB* is repressed by the binding of *korB* to common sequences of each promoter. *korA* represses both the *kilA* and transfer functions indicating that control of conjugative transfer is mediated in part by the action of the *kil/kor* system (Stein *et al.*, 1989).

Section II. Bacteriocins, colicins, and microcins

Bacteriocins are a general class of macromolecules produced by certain bacteria that are able to arrest the growth or kill the same or other types of bacteria. Bacteriocins are proteins that diffuse from one organism to another and cause a one-hit response within the susceptible recipient. Although Rodriguez and Iyer (1981) have shown that the killing of *K. oxytoca* by bacteria carrying pCU1 is not mediated by a diffusible product, it is worth comparing this effect to those of bacteriocins. A three part mechanism has been proposed for killing by bacteriocins: 1) a stimulus must occur for initiation of bacteriocin release, 2) a receptor must be available for the molecule to attach, and 3) a biochemical target must be present that is affected by the bacteriocin and this event causes cell death (Luria, 1973). Bacteriocins are produced by Gram-negative as well as Gram-positive bacteria, although there is very little cross-reactivity. Each utilizes a unique method of killing, generating a wide variety of mechanisms for killing bacteria. The classification of bacteriocins is largely based upon the species of bacteria that are affected (Reeves, 1972).

Colicins are a specific class of bacteriocins which are bacteriocidal for *E. coli* and closely related members of the *Enterobacteriaceae* (reviewed by Hardy, 1975). Most colicins are released from the cell and able to diffuse through the surrounding media until contact is made with another cell. Others, however, are not released and

remain cell bound. These are found only rarely. The genetic determinants for colicin production and immunity are all localized to bacterial plasmids. Colicins are quite large with respect to other types of antibiotics and usually cannot penetrate the bacterial cell wall. Initially, colicins react with receptors on the cell surface which normally act as receptors for metabolites to be transported into the cell. Some colicins may be split into N-terminal and C-terminal fragments at the cell surface, the N-terminal (a hydrophobic moiety interacting with the receptor) left behind at the cell surface and the C-terminal (a highly basic peptide) free to enter the cell to act as a nuclease (Hardy, 1983). Once established, colicins exhibit three main mechanisms for killing: 1) disruption of the cytoplasmic membrane, 2) degradation of DNA and RNA by nuclease activity and inhibition of cell division, and 3) inhibition of protein synthesis (Broda, 1979).

Microcins are low molecular weight amino acid-peptide antibiotics produced and excreted by members of the *Enterobacteriaceae*. Microcins differ from colicins only by size. Colicins have a molecular weight ranging from 50,000 to 90,000 daltons whereas microcins are of a molecular weight below 40,000 daltons (Asensio *et al.*, 1976). Currently, microcins are classified into five types (A, B, C, D, and E) by cross-immunity, biochemical, and genetic criteria (Baquero and Moreno, 1984). All microcin types have been shown to be diffusible and associated with bacterial plasmids, most of which are conjugative (Baquero *et al.*, 1978). Types A through D

were isolated from various host strains, while type E were isolated from strains of *K. pneumoniae*. Microcins of each group have a different mode of action. Type A microcins inhibit metabolic enzymes. For example, microcin A15 blocks protein synthesis by mimicking the allosteric inhibition of L-methionine and S-adenyl methionine, thus blocking the first enzyme in the methionine biosynthetic pathway. Type B specifically inhibits DNA replication. The bacterial DNA is degraded and the SOS response is invoked (Herrero and Moreno, 1986). Type C may stall protein synthesis by mimicking some signal peptides. Type D impair the cell energy-generating system by inhibiting proline transport and depleting the ATP pool within susceptible cells. This is accomplished by perturbing the transmembrane electrical potential. Type E microcins, identified from strains of *K. pneumoniae*, are hydrophobic basic peptides that are mainly produced during the early stationary phase of growth. Their synthesis is known to be severely reduced in richer media. The type E microcins also seem to be active in disrupting the transmembrane electrical potential (Baquero and Moreno, 1984). None of these microcins have yet been genetically characterized.

By far, the most work has been on the study of microcin B17. Microcin B17 is a hydrophobic peptide of approximately 3,200 daltons. The genetic determinants for microcin B17 and immunity are encoded by pMccB17, an IncFII conjugative plasmid of 70 kb. Microcin B17 production has been localized to a 3.0 - 3.5 kb

segment and another 1.0 - 2.0 kb of DNA is required for immunity. Complementation studies have shown that at least four genes (*mcbA*, *mcbB*, *mcbC*, and *mcbD*) are involved in microcin B17 synthesis (San Millan, *et al.*, 1985a; San Millan, *et al.*, 1985b). Sequence analysis of the microcin B17 region has confirmed the transcriptional units and predicts the size of the McbA protein to be 69 amino acids in length (6,013 daltons) (Genilloud *et al.*, 1989). The inactive McbA protein is processed by removal of the first 26 amino acids from its N-terminus. An interesting property of microcin B17 is that the active protein contains sixty percent glycine (Davagnino, *et al.*, 1986).

The mode of action of microcin B17 is to inhibit DNA elongation and has been shown to invoke the SOS response to DNA damage in *E. coli* (Herrero and Moreno, 1986). It is not yet known if microcin B17 interacts directly with any DNA replication proteins for inhibition of DNA replication.

Interestingly, production of microcin B17 in normal amounts requires at least three chromosomally located genes one of which is *ompR*, the positive regulator of transcription for the genes encoding the major outer membrane porins, OmpC and OmpF (Hernandez-Chico *et al.*, 1986). Microcin B17 is not expressed in cells during early and mid-exponential growth, but is expressed as cultures enter stationary phase. This has been shown to be controlled by the level of transcription initiation originating from a promoter upstream of the structural gene for microcin B17

(*mcbA*). Starvation for nitrogen, phosphate, or carbon all induce expression from this promoter (Connell *et al.*, 1987). *mcbA* is thus an example of a plasmid gene which utilizes a chromosomally encoded regulatory molecule for transcriptional control. Two other promoters have been identified within the microcin B17 sequence that initiate transcription in opposite directions (Genilloud *et al.*, 1989).

Studies of the immunity region for microcin B17 have shown that immunity is determined by three open reading frames, encompassing the *mcbE*, *mcbF*, and *mcbG* genes. Immunity to microcin B17 involves two different mechanisms. One, encoded by *mcbE* and *mcbF*, has been proposed to act as a membrane "pump" for export of microcin B17 from the cell. The predicted *mcbE*-encoded product has properties of a membrane protein as shown by hydrophobicity profile and that of *mcbF* has properties of an ATP-binding protein as it contains a possible nucleotide binding site of Gly-X-X-X-X-Gly-Lys-Thr, where X designates any amino acid. The other mechanism of immunity involves partial resistance of the cell to microcin B17 encoded by *mcbG*. In the absence of *mcbG*, cells grow at a slower rate, show pronounced SOS induction, plasmid loss, and cell filamentation. All of these are the result of microcin B17 activity within the cell (Garrido *et al.*, 1988). Recently, del Castillo *et al.* (1990) have cloned an *E. coli* chromosomal gene which suppresses a *mcbG* mutation when on a multicopy plasmid vector. This gene, designated *mprA* (for microcin production regulation), is believed to encode a 19 kDa protein that

blocks transcription from the promoter located upstream from *mcbA* as well as other seemingly unrelated chromosomal genes. It is possible that this is accomplished by binding of the *mprA* protein to transcriptional regulatory regions near *E. coli* chromosomal loci involved in growth rate dependent expression.

Another, microcin C7, has also been genetically characterized. Microcin C7 is a linear heptapeptide which blocks protein synthesis. The determinants for Microcin C7 and immunity are located on the 43 kb conjugative IncX plasmid pMccC7. Four transcriptional units (α , β , γ , and δ) for the production of microcin have been localized within a 5 kb stretch of DNA by transposon mutagenesis. All four regions seem to be involved in immunity. Tn5 insertion mutations mapped in the γ region did not produce microcin and expressed weak immunity. The γ region may therefore encode the structural gene for microcin C7. Like microcin B17, microcin C7 is also induced when cells reach stationary phase and it has been shown that the chromosomal regulator gene *mprA* also inhibits production of microcin C7 (del Castillo, 1990). However, in contrast to microcin B17, *ompR* does not affect the expression of microcin C7. (Novoa *et al.*, 1986). Therefore, it appears that microcin C7 and microcin B17 have different killing mechanisms, but are related by their ability to be expressed during a specific stage of a developmental growth pattern. Little information is available at this time on the signals that trigger expression of genes related to bacterial growth.

Section III. Molecular biology of DNA transfer during bacterial conjugation

Because of the technical necessity to transfer *kikA* and *kikB* into *Klebsiella* for the detection of the Kik⁺ phenotype, a detailed description of a current model of molecular interactions before, during, and after bacterial conjugation is included. Introduction of *kikA* into *K. oxytoca* by transformation has not been demonstrated to affect the viability of *Klebsiella* cells due to the relatively low numbers of transformants. In order to discuss the possible mechanisms of the Kik⁺ phenomenon common to IncN plasmids, it is important to review what is known about conjugative transfer of DNA. Extensive information is available on F plasmid transfer since this system has been studied in some detail. It is for this reason that F plasmid is held as the model system for plasmid conjugation. Much of this knowledge also holds true for other incompatibility group plasmids, although the details may not be strictly adhered to. Differences between specific plasmids are common and therefore considered. The following is a description of the conjugative system of IncF plasmids.

Bacterial Conjugation

Conjugation can be divided into two major processes: mating pair formation and DNA transfer. First, the formation of mating pairs must occur. The plasmid-encoded pili play a very important role in contact with recipient cells. Thin flexible

pili and some thick flexible pili are equally good at conjugation in broth media and on solid media. Short, pointed, and rigid pili, such as those encoded by IncN, IncP, and IncW group plasmids, are conjugatively more efficient on solid surfaces (Bradley, 1981) or in a physical environment that provides an increased surface-to-volume ratio (Singleton, 1983). Considerable evidence exists that pilus-mediated contact is necessary for mating pair and aggregate formation but that subsequent cell contact may be the actual instigator of DNA transfer through the cell membrane (Achtman and Skurray, 1977; Achtman, 1978). The ends of the pili contact receptor sites within the recipient cell membrane. Bacterial cells deficient for the major outer membrane protein of *ompA* are resistant to certain phages, colicins L and K, and serve as poor recipients for F transfer suggesting that the protein encoded by *ompA* acts as the receptor for F pili (Achtman and Skurray, 1977).

Second, DNA must be transferred from the donor cell to the recipient. After contact has been made between the tip of the pilus and the recipient cell, the pilus retracts until the cell membranes of both cells come into close contact. At this point, the wall-to-wall contact results in an intermembrane channel. A mating signal from the recipient then stimulates the initiation of DNA transfer. The exact trigger for the donor to begin the transfer mechanism is unknown (Ippen-Ihler and Minkley, 1986). A single plasmid strand is nicked by a plasmid encoded enzyme at

the origin of transfer (*oriT*). Transfer of F plasmid DNA is initiated by recognition of *oriT*. One model of transfer involves the recognition of *oriT* and binding of proteins to form a "transfer complex" (Willetts and Skurray, 1987). After nicking, the 5' terminal end of the single-stranded plasmid DNA is transferred to the recipient cell. Only one strand of DNA is transferred. Transfer is oriented such that the transfer region (*tra*) is transferred last. (Willetts and Wilkins, 1984). Once the single-stranded DNA enters the recipient cell, the newly transferred strand of DNA begins replication.

DNA synthesis in both the donor and recipient is essential to plasmid DNA transfer. Conjugative DNA synthesis can be thought of as a two-fold process: donor cell DNA synthesis (DCDS) and recipient cell DNA synthesis (RCDS). DCDS requires host encoded DNA polymerase III and an RNA priming system. Within the recipient cell, DNA processing involves both RCDS and circularization of the plasmid DNA. For the IncF plasmids, the order is RCDS first and circularization second. This may not be true for other plasmids. The current model of recircularization is that during the nicking event, two polypeptides become covalently attached to the 5' and 3' termini of the DNA. Reassociation of these two peptides within the recipient cell connects the ends (Ippen-Ihler and Minkley, 1986). RCDS also proceeds by host DNA polymerase III. In contrast to DCDS, the RNA priming system of RCDS is apparently driven primarily by plasmid-encoded DNA primase

supplied by the donor cell. Some conjugative plasmids also encode a DNA primase. Little is known about the RNA priming systems utilized, the removal of primer, gap filling, and ligation of the termini of the newly synthesized DNA strand in DCDS and RCDS (Willetts and Wilkins, 1984). In review, plasmid DNA processing during conjugation involves several steps: i) mating pair formation, ii) nicking at *oriT*, iii) initiation of DNA transfer at *oriT*, iv) strand separation, v) strand transfer, vi) recircularization, and vi) initiation of vegetative replication of plasmid DNA.

From this discussion of conjugative DNA transfer, it is apparent that plasmid-encoded proteins may also be transferred from donor to recipient during conjugation. Mentioned above are proteins involved in initiating transfer at *oriT*. The presumptive conjugative DNA-protein complex may provide the vehicle for transfer of proteins as well as DNA. It is likely that single-stranded DNA binding proteins are required to promote chain elongation and perhaps protect the DNA from nuclease activity. F plasmid contains a gene, *ssf*, that encodes a protein with extensive homology to the *E. coli* single-stranded DNA binding protein. The *ssf* gene product may participate in conjugative DNA metabolism during RCDS. If so, this protein would have to be transported to recipient cells during conjugation since F plasmid gene expression is not required for RCDS.

It has been shown that a plasmid-encoded DNA primase is transferred during conjugative transfer of IncI plasmid ColIb-P9 (Chatfield and Wilkins, 1984). This

plasmid carries a gene designated *sog* due to its ability to suppress a *dnaG* mutation, *dnaG* being the *E. coli* gene responsible for producing the primase required for chromosomal DNA synthesis. Mutations within the *sog* region show some deficiency in conjugative transfer function of the plasmid. This indicates that the primase encoded by *sog* is somehow involved in conjugative DNA synthesis. The *sog* primase is apparently produced within the donor cell and transferred to recipient cells while bound to the single-stranded DNA. It also appeared that two *sog* peptides are among a group of proteins that are transferred to the recipient cell during conjugation providing the most direct evidence for the proposed conjugative DNA-protein transfer complex (Merryweather *et al.*, 1986a; Merryweather *et al.*, 1986b). It is estimated that as many as 500 protein molecules of *sog* are passed from donor to recipient during a single round of mating (Rees and Wilkins, 1989).

Two phenomena resulting in bacteriocidal effects associated with bacterial conjugation are zygotic induction and lethal zygotis. In a general sense, zygotic induction can be thought of as the bringing about of a particular function upon zygote formation. The killing of *K. oxytoca* can thus be a result of either the transfer of a substance which provides killing or the transfer of a substance lacking the ability to inhibit killing. A model for an event resulting from lack of an inhibitory factor would then be the induction of prophage λ . Although the Kik phenomenon is not the result of a phage infection, it may be useful to keep such a mechanism

in mind.

The mechanism of lethal zygosis is particularly intriguing. Conjugative behavior of F transfer and death of *E. coli* may prove to be similar to the mechanism by which *K. oxytoca* cells are killed during conjugation.

Molecular mechanism of zygotic induction

Zygotic induction is a phenomenon in which conjugative recipient cells of *E. coli* are killed as a result of induction of the bacteriophage λ lytic cycle. Such an occurrence happens readily after a high frequency of recombination (Hfr) donor cell is mated with an F^- recipient cell. In the case of Hfr mating, the F plasmid has become integrated into the bacterial chromosome. Conjugative transfer of part of the bacterial genome is accomplished by the same mechanism as F plasmid transfer alone, leading to a high frequency of genetically recombinant bacteria. If the donor cell contains a chromosomally integrated copy of phage λ , the phage genome can be transferred. Transfer of the prophage genome unrepressed for lytic functions produces infection from within to kill the cells. This phenomenon occurs only if the recipient does not have λ integrated in the host genome (Jacob and Wollman, 1961). The study of zygotic induction and of the mechanism by which λ chooses the lysogenic cycle rather than the lytic cycle has led to the discovery of a repressor that binds to particular regions of the phage DNA. Binding of repressor, found to

product of the phage λ *cI* gene, prohibits expression of genes involved in the lytic cycle. Recipient cells which lack the repressor are then susceptible to the production of λ phage infection (Lewin, 1977; Ptashne, 1978).

The phenomenon of lethal zygotis

When *E. coli* cells containing an integrated F plasmid (Hfr) are mated with F^- cells, a percentage of the recipient population become non-viable. This phenomenon of lethal zygotis is not the result of prophage induction because culture filtrates and supernatant fluids from donors do not produce the effect. In addition, this phenomenon is unlike that of zygotic induction since the presence or absence of bacteriophage λ does not affect lethality. Direct cell-to-cell contact that occurs during conjugation is required for killing. After two hours of mating, 95% of recipients are killed. Survivors sometimes carry the F plasmid and can act as normal donors in subsequent matings; however, they are immune to further killing. Interestingly, F^+ donors do not kill recipients to the extent of Hfr donors. Only rarely do F' donors show the same effect. Also, recipient cells in the late stationary phase of growth seem to be more susceptible than rapidly growing cells. The killing effect is more pronounced when donors are grown in enriched media and are used in matings when in the logarithmic phase of growth. These observations have led to a hypothesis that metabolic activity in the donors provides efficient conjugative

mechanisms, but that metabolically active recipients are somewhat resistant to the lethal effects. The important implication is that mating damages recipients and that they may not recover if unable to repair the damage quickly (Skurray and Reeves, 1973a).

Studies of the physiology of recipients shows that cells subjected to Hfr mating have lost the ability to complete energy dependent processes and that this is most likely due to disruption of the cytoplasmic membrane (Skurray and Reeves, 1973b). The possible mechanism of lethal zygosis would then be either interference with energy-generating mechanisms or disruption of the permeability barrier. A role of conjugation in killing could be physical disruption of the membrane. However, $F^+ \times F^-$ matings under the same conditions do not show lethal zygosis as do Hfr $\times$ F^- matings. It is possible that events during normal conjugative transfer include a step whereby the recipient cell is physiologically as well as physically immobilized. Two current hypothetical reasons for cell death are 1) high ratios of donor:recipient produce multiple hits whereby recipients are unable to recover, or 2) transfer of large amounts of DNA (as with Hfr donors) prolongs the event that immobilizes recipients and they cannot recover. Thus, $F^+ \times F^-$ matings may undergo the same event, but that lethal zygosis is not observed due to a very short immobilization period that allows recovery of recipients. The events that lead to lethal zygosis remain obscure.

Immunity to lethal zygosis is observed when recipients of F conjugation already contain a copy of the F plasmid. In matings of Hfr x F⁻, survivors are no longer sensitive to lethal zygosis. The resistance is due to transfer of F to recipients. Two mechanisms for immunity have been postulated, one that involves surface exclusion of F pili from the recipient membrane receptor sites thus prohibiting conjugation, and one involving two plasmid borne loci (Skurray and Reeves, 1974).

Skurray and Reeves (1974) localized *ilzA* and *ilzB* by deletion analysis of several Hfr donor strains. Studies of donors carrying deletions of F showed a reduction of immunity and led them to propose these regions are required for an immunity distinct from that of surface exclusion. Further studies by Ou (1980) showed that both types of immunity could be attributed to surface exclusion. The region designated as *ilzA* was found to be *traS*, a transfer gene encoding a 16.9 kDa inner membrane protein, and that *ilzB* is most likely *traT*, a transfer gene encoding a 26 kDa outer membrane lipoprotein (Achtman *et al.*, 1977; Ou, 1980). Both *traS* and *traT* cause the cell to act as poor recipients in F mediated conjugation. *traT* may prevent mating pair formation by masking the receptor site for F pili, while *traS* may prohibit DNA transfer (Willetts and Skurray, 1987).

From the previous description of the killing phenomena associated with bacterial plasmids, it is apparent that many plasmids possess functions that are lethal to the host cell. The possible relatedness of these functions to the killing of *K. oxytoca* by plasmid pCU1 has prompted this investigation into the mechanism of killing. Since the original description of the killing of *K. oxytoca* by Rodriguez and Iyer in 1981, no other reports of a plasmid-borne phenomenon of this type for other incompatibility plasmids have occurred in the literature. The Kik phenomenon is unique in this respect. Only one mechanism of killing cells by conjugation, that of lethal zygosis, appears to be somewhat similar to the Kik phenomenon. However, to date, no chromosomally linked or plasmid associated genes have been attributed directly to lethal zygosis. Also, the mechanism by which *E. coli* recipient cells die after mating remains obscure. This thesis is focused on the *kikA* region and provides the groundwork for an understanding of the mechanism and role of the Kik phenotype.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used in this study are listed in Table 1. *K. oxytoca* strains used are listed in Table 2. All bacterial species were kept in storage by suspension of log phase cells in 500 μ l TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) and mixing with 1.5 ml 80% glycerol in TE buffer pH 8.0. The glycerol cultures were then stored at -20°C.

Bacteriophages

Bacteriophage vectors M13mp18 and M13mp19 were purchased from Bethesda Research Laboratories, Bethesda, Maryland, U.S.A. Replicative-form (RF) DNA from each was prepared by the large scale plasmid isolation procedure described elsewhere in the Materials and Methods section of this thesis. *E. coli* strain DH5 α F' was transfected with M13 RF DNA by the standard CaCl₂ method described for the transformation of competent cells.

Plasmids

Vector plasmids used and recombinant plasmid constructs created in this study are listed in Table 3.

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

Strain	Genotype/Phenotype*	Source or Reference
J5	F ⁻ <i>pro-22 met</i> ⁻	Datta, 1975
HB101	F ⁻ <i>hsdR17 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i> λ ⁻	Bolivar <i>et al.</i> , 1977
C600	F ⁻ <i>thi thr leuB6 lacY1 tonA21 supE44</i> λ ⁻	Bachmann, 1972
C600Rif ^r	Same as C600 but resistant to 200 μ l/ml rifampicin	This laboratory
C600Nal ^r	Same as C600 but resistant to 60 μ l/ml nalidixic acid	This laboratory
DH5	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1</i> λ ⁻	Hanahan, 1985
DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 ϕ80 dlacZ M15 Δ(lacZYA-argF)U169</i> λ ⁻	Jessee, 1986
DH5 α F'	F' <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 ϕ80 dlacZ M15 Δ(lacZYA-argF)U169</i> λ ⁻	Liss, 1987
MC1061	<i>araD139 Δ(ara, leu)7697 lacIPOZY galU galK hsr hsm strA</i>	Casadaban, 1980

Table 1. (cont.) List of *E. coli* strains used

Strain	Genotype/Phenotype*	Source or Reference
MC1061Rif ^r	Same as MC1061 but resistant to 200 μ l/ml rifampicin	This laboratory
C2107	<i>polA12 mnh his</i>	Krevolin <i>et al.</i> , 1985
C2107Rif ^r	Same as C2107 but resistant to 200 μ l/ml rifampicin	This laboratory

* Nomenclature of abbreviated strain designations taken from Bachmann (1990): *ara*, arabinose; *arg*, arginine; *endA*, DNA-specific endonuclease I; *gal*, galactose; *gyr*, gyrase; *his*, histidine; *hsd*, host-specificity; *hsm*, host-specificity modification; *hsr*, host-specificity restriction; *lac*, lactose; *leu*, leucine; *met*, methionine; *pol*, polymerase; *pro*, proline; *rec*, recombination; *rel*, relaxed; *mnh*, RNase H; *rps*, ribosome protein (small); *srl*, sorbitol; *str*, streptomycin; *sup*, suppressor; *thi*, thiamin; *thr*, threonine; *ton*, T-one; *xyl*, xylose.

Table 2. List of *K. oxytoca* strains used

Strain	Genotype/Phenotype	Source or Reference
UN	prototroph, same as <i>K. pneumoniae</i> M5a1	McNeil <i>et al.</i> , 1981
UN2979	<i>hisD4226 lacZ4001</i> galactose resistant	McNeil <i>et al.</i> , 1981
UN4102	<i>hisD lacZ recA56 srl::Tn10</i> galactose resistant	McNeil <i>et al.</i> , 1981

Table 3. List of plasmids used

Plasmid	marker(s)	Description	Source or Reference
pACYC184	Cm,Tc	cloning vector	Chang and Cohen, 1978
pBR322	Ap,Tc	cloning vector	Bolivar <i>et al.</i> , 1977
pJF118EH	Ap	expression vector	Fürste <i>et al.</i> , 1986
pJF118HE	Ap	expression vector	Fürste <i>et al.</i> , 1986
pUC7	Ap	cloning vector	Vieira and Messing, 1982
pUC18	Ap	cloning vector	Yanisch-Perron <i>et al.</i> , 1985
pUC19	Ap	cloning vector	Yanisch-Perron <i>et al.</i> , 1985
pUC1318	Ap	cloning vector	Kay and McPherson, 1987
pUC1813	Ap	cloning vector	Kay and McPherson, 1987
pUC1318-Kan/ <i>oriT</i>	Ap,Km	cloned <i>oriT</i> _{RK2}	This study
pUC1813-IncN <i>oriT</i>	Ap	cloned <i>oriT</i> _{PCU1}	This study
RK2	Ap,Km,Tc	parental plasmid	Meyer <i>et al.</i> , 1975
RP4	Ap,Km,Tc	same as RK2	Datta, 1975
pS-a	Cm,Km, Sm,Su	parental plasmid	Datta, 1975
R144 <i>drd3</i>	Tc, Km	parental plasmid	Datta, 1975

Table 3. List of plasmids used (cont.)

Plasmid	marker(s)	Description	Source or Reference
R45	Ap,Su,Tc	parental plasmid	Datta, 1975
R46	Ap,Sm, Su,Tc	parental plasmid	Datta, 1975
R48	Ap,Sm, Sp,Su,Tc	parental plasmid	Datta, 1975
pTM4245	Ap,Sm,Km	parental plasmid	Babudri and Monti-Bragadin, 1977
pKM101	Ap	deletion of R46	McCann <i>et al.</i> , 1975
pCU1	Ap,Sm,Sp	parental plasmid	Konarska-Kozłowska and Iyer, 1981b
pCU31	Ap	deletion of pCU1	Konarska-Kozłowska and Iyer, 1983
pCU66	Ap,Sm,	Tn5 in pCU1	Thatte <i>et al.</i> , 1985a
pCU101	Cm	pCU1 <i>tra</i> in pACYC184	Thatte and Iyer, 1983
pCU105	Cm	pCU1 <i>tra</i> in pACYC184	Thatte and Iyer, 1983
pCU109	Cm	pCU1 <i>tra</i> in pACYC184	Thatte and Iyer, 1983
pCU167	Ap,Sm, Sp,Km	Tn5 in pCU1	Thatte <i>et al.</i> , 1985a

Table 3. List of plasmids used (cont.)

Plasmid	marker(s)	Description	Source or Reference
pCU171	Ap,Sm, Sp,Km	Tn5 in pCU1	Thatte <i>et al.</i> , 1985a
pCU184	Ap,Sm, Sp,Km	Tn5 in pCU1	Thatte <i>et al.</i> , 1985a
pCU1000	Ap	<i>kikB</i> from pCU31 in pBR322	This study
pCU1066	Ap	<i>kikB</i> from pCU109 in pUC7	This study
pCU1106	Cm	<i>kikA</i> from pCU171 in pACYC184	This study
pCU1107	Cm	<i>SalI</i> deletion of pCU1106	This study
pCU1108	Cm	IncN <i>oriT</i> cloned onto pCU1107	This study
pCU1110	Cm	<i>Bam</i> HI deletion of pCU1108	This study
pCU1110/25	Cm	IncN <i>oriT</i> cloned onto pCU1125	This study
pCU1110/27	Cm	IncN <i>oriT</i> cloned onto pCU1127	This study
pCU1110/29	Cm	IncN <i>oriT</i> cloned onto pCU1129	This study

Table 3. List of plasmids used (cont.)

Plasmid	marker(s)	Description	Source or Reference
pCU1110/36	Cm	IncN <i>oriT</i> cloned onto pCU1136	This study
pCU1110/49	Cm	IncN <i>oriT</i> cloned onto pCU1149	This study
pCU1110/29- Δ 21	Cm	<i>Bal31</i> deletion of pCU1110/29	This study
pCU1125	Cm	<i>Bal31</i> deletion of pCU1107	This study
pCU1127	Cm	<i>Bal31</i> deletion of pCU1107	This study
pCU1129	Cm	<i>Bal31</i> deletion of pCU1107	This study
pCU1136	Cm	<i>Bal31</i> deletion of pCU1107	This study
pCU1139	Cm,Km	Kan/ <i>oriT</i> cloned onto pCU1107	This study
pCU1140	Cm,Km	Kan/ <i>oriT</i> cloned onto pCU1107	This study
pCU1149	Cm	<i>Bal31</i> deletion of pCU1107	This study
pPH0	Cm	composite from pCU1110-29 and pCU1110-49	This study

Table 3. List of plasmids used (cont.)

Plasmid	marker(s)	Description	Source or Reference
pPH0/1	Cm	<i>Bal31</i> deletion of pPH0	This study
pPH0/5	Cm	<i>Bal31</i> deletion of pPH0	This study
pPH0/6	Cm	<i>Bal31</i> deletion of pPH0	This study
pPH0/8	Cm	<i>Bal31</i> deletion of pPH0	This study
pPH0/11	Cm	<i>Bal31</i> deletion of pPH0	This study
pPH3	Ap	<i>kikA</i> fragment in pJF118EH	This study
pPH4	Ap	<i>kikA</i> fragment in pJF118HE	This study
pNH142	Cm,Km	Tn5 <i>lac</i> in pCU109	This study
pNH142 Δ K5	Cm,Km	<i>KpnI</i> deletion of pNH142	This study
pUC19-66A	Ap,Km	<i>SalI</i> fragment of pCU66 in pUC19	This study
pUC19-167A	Ap,Km	<i>SalI</i> fragment of pCU167 in pUC19	This study
pUC19-184A	Ap,Km	<i>SalI</i> fragment of pCU184 in pUC19	This study

Table 4. List of bacteriophages used

Phage	Description	Source or Reference
M13mp18	cloning vector	Yanisch-Perron <i>et al.</i> , 1985
M13mp19	cloning vector	Yanisch-Perron <i>et al.</i> , 1985
M13-KA108	<i>Bam</i> HI- <i>Xho</i> I <i>kikA</i> fragment of pPH0/1 cloned into the <i>Bam</i> HI- <i>Sal</i> I site of M13mp18	This study
M13-KA201	<i>Bam</i> HI- <i>Xho</i> I <i>kikA</i> fragment of pPH0/1 cloned into the <i>Bam</i> HI- <i>Sal</i> I site of M13mp19	This study
M13-KA305	4.0 kb <i>Eco</i> RI- <i>Hind</i> III fragment of pUC19-184A cloned into M13mp18	This study
M13-KA412	4.0 kb <i>Eco</i> RI- <i>Hind</i> III fragment of pUC19-167A cloned into M13mp18	This study

Growth conditions, media, and antibiotics

E. coli and *K. oxytoca* strains were grown at 37°C overnight in LB (Maniatis, *et al.*, 1982): 10.0 g/l tryptone (Difco), 5.0 g/l yeast extract (Difco), and 10.0 g/l NaCl. LB medium was used as broth or as agar plates prepared by addition of 16.0 g/l Difco bacto agar. For production of infectious M13 particles, 2xYT broth: 16.0 g/l tryptone, 10.0 g/l yeast extract, and 5.0 g/l NaCl was used for growing the host strain. Plaques were formed by transfecting *E. coli* DH5 α F' and inoculating seeded soft H-agarose: 10.0 g/l tryptone, 8.0 g/l NaCl, and 8.0 g/l agarose that was overlayed onto an H-agar base: 10.0 g/l tryptone, 8.0 g/l NaCl, 16.0 g/l agar. For bacterial matings with *K. oxytoca* as recipients, glucose M9 minimal media (Miller, 1972) was supplemented with 100 μ g/ml L-histidine. Minimal media was prepared as follows: a 5x stock of minimal salts containing 30.0 g/l Na₂HPO₄, 15.0 g/l KH₂PO₄, 2.5 g/l NaCl, and 5.0 g/l NH₄Cl (pH 7.0) was autoclaved separately from and later added to a solution of agar in H₂O to bring the final concentration of agar to 1.6% w/v and the salt concentration to 1x. After tempering the agar solution to 45°C, glucose was added to a final concentration of 0.2% w/v, MgSO₄ · 7H₂O to a final concentration of 1.0 mM, CaCl₂ to a final concentration of 0.1 mM, and L-histidine to a final concentration of 100 μ g/ml. For *E. coli* strains, casamino acids (Difco) were added to a final concentration of 200 μ g/ml. For the induction studies,

IPTG was added to minimal media to give a final concentration of 0.1 M.

Antibiotics purchased from Sigma Chemical Corp., St. Louis, Missouri, U.S.A. were added to either LB or M9 minimal agar from filter sterilized stock solutions to produce a final concentration of: 200 $\mu\text{g/ml}$ carbenicillin, 50 $\mu\text{g/ml}$ chloramphenicol, 10 $\mu\text{g/ml}$ tetracycline, 100 $\mu\text{g/ml}$ kanamycin sulfate, 200 $\mu\text{g/ml}$ rifampicin and 60 $\mu\text{g/ml}$ nalidixic acid for *E. coli* strains. *K. oxytoca* transformants were maintained on media containing 600 $\mu\text{g/ml}$ carbenicillin.

Extraction of plasmid DNA

The large scale method of isolating purified plasmid DNA was a modified version of the Triton X-100 method of Clewell and Helinski (1969) incorporating a PEG precipitation step described by Humphreys *et al.* (1975). *E. coli* cells containing the plasmid to be isolated were inoculated onto 5-10 selective LB plates from a primary culture grown in liquid media. After two days of growth at 37°C, each plate was flooded with 3 ml of LB broth. The cells were scraped together with a bent glass rod, and pooled into an Sorval SS34 centrifuge tube. The tubes were centrifuged at 3,000 $\times$ g (5,000 rpm) for 15 min. The cell pellet was resuspended in 10 ml of sucrose buffer (50 mM Tris; 1 mM EDTA; 25% w/v sucrose; pH 8.0) and placed on ice for 10 min. 1.0 ml of 10 mg/ml lysozyme in 0.25 M EDTA, pH8.0 was mixed in by swirling and the tube placed on ice for 10 min. 4.0 ml of Triton X-100

solution (50 mM Tris; 60 mM EDTA; 2% v/v Triton X-100; pH 8.0) was added and the cells placed at 65°C for 15-30 min. until viscous. The tubes were centrifuged at 40,000xg (18,000 rpm) for 45 min. The clear liquid supernatant was transferred to a clean SS34 tube and 5.0 ml of PEG-NaCl solution (40% w/v PEG; 2 M NaCl) was added. After the DNA precipitated overnight at 4°C, the tubes were centrifuged at 8,000xg (7,000 rpm) for 10 min. The supernatant was decanted and two centrifuge tubes washed with 1.7 ml sterile distilled water each, were then pooled to make a total volume of 3.4 ml. 4.00 g of CsCl was dissolved into the liquid and 0.8 ml of 4 mg/ml ethidium bromide was added. The DNA solution was transferred to a Beckman Quickseal polypropylene ultracentrifuge tube and the tube sealed with a Beckman heat-sealer. The tubes were spun in a Beckman VTi80 rotor in a Beckman model L8-80 ultracentrifuge at 300,000xg for 16-20 hr. at 15°C. After centrifugation, the tubes were carefully removed and placed in front of a longwave UV light source to visualize the DNA bands. A 3 ml syringe fitted with a 21 gauge needle was used to retrieve the lower band. Ethidium bromide was removed from the DNA by three extractions with cold isoamyl alcohol and CsCl removed by dialysis against TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0). The DNA was precipitated by addition of 0.25 volumes of 10 M ammonium acetate and 2.5 volumes of pre-chilled absolute ethanol at -20°C overnight. The DNA was centrifuged in an eppendorf table top centrifuge (approx. 14,000xg) for 15 min. The

pellet was washed once with 100 μ l of 70% v/v ethanol and again centrifuged before drying in a vacuum dry air oven at 50°C for 10 min. Each DNA pellet was dissolved in 20 μ l of sterile distilled water. All DNA samples were stored frozen at -20°C.

For analysis of plasmid DNA from a number of clones, it was necessary to isolate DNA by a small scale method. The mini-prep method used was a modified version of the alkaline lysis technique of Birnboim and Doly (1979). *E. coli* cells containing plasmids were grown in 5.0 ml liquid media containing the appropriate antibiotic. The cell cultures were chilled on ice for 10 min. prior to centrifugation at 8000xg for 5 min. in a clinical table top centrifuge. The cell pellet was resuspended in 100 μ l of TE buffer (25 mM Tris; 10 mM EDTA; pH8.0), transferred to an eppendorf tube, and placed on ice for 10 min. 200 μ l of lysis solution (0.2 M NaOH; 1% w/v SDS) prewarmed to 60°C was injected into the cell suspension and the layers mixed by inversion. The tubes were placed back on ice for 15-20 min. until lysis was complete. This was apparent by clouding and increased viscosity within the tube. 150 μ l of 3 M sodium acetate, pH 4.8 was then added and the contents mixed by flicking the tube. The tubes were again placed on ice for 30-60 min. The tubes were then spun in an eppendorf centrifuge (approx. 14,000xg) for 15 min. and the clear supernatant was transferred to a fresh eppendorf tube. 125 μ l of 10 M ammonium acetate was added and the DNA

precipitated by adding 1.0 ml of prechilled absolute ethanol and placing the tubes at -20°C overnight. Precipitated plasmid DNA was recovered by spinning in an eppendorf centrifuge (approx. $14,000\times g$) for 30 min. and resuspending the white pellet in $200\ \mu\text{l}$ of 70% v/v ethanol through three wash cycles. The white pellet remaining was then dried in a vacuum dry air oven at 50°C for 10 min. and the DNA dissolved in $20\ \mu\text{l}$ of sterile distilled water.

Frequently, restriction fragments from either purified or mini-prep DNA were recovered from an agarose gel for subcloning. Isolation of DNA from agarose gels was accomplished by binding the desired fragment of DNA to a silica matrix in the presence of NaI (Vogelstein and Gillespie, 1979). Restricted DNA was first electrophoresed through an agarose gel buffered with a Tris acetate solution (40 mM Tris; 20 mM sodium acetate; 2 mM EDTA; pH 7.6). The gel was stained in ethidium bromide and exposed to longwave UV light to locate the desired DNA fragment. Sizing of the DNA fragments was accomplished by comparison against a 1 kb DNA ladder composed of yeast plasmid polymers purchased from Bethesda Research Laboratory, Bethesda, Maryland, U.S.A. (Hartley and Donelson, 1980) run in an adjacent well. The correct DNA fragment was excised from the gel by slicing with a razorblade and transferred to an eppendorf centrifuge tube. 2.5 volumes of saturated NaI solution was added and the tube incubated at 60°C for 5-10 minutes until all traces of agarose had dissolved. $5\ \mu\text{l}$ of silica matrix (purchased as a

GENECLEAN kit from Bio101 Inc., La Jolla, California, U.S.A.) was added and the suspension incubated at 20°C for 10 min. The tube was then centrifuged for 10 sec. (approx. 14,000xg) in an eppendorf centrifuge. The matrix pellet was washed with 200 µl of NEW buffer solution (10 mM Tris; 100 mM NaCl; 1 mM EDTA; 50% v/v ethanol) through three wash cycles. After the final spin, the pellet was dried in a vacuum dry air oven at 50°C for 10 min. and the DNA dissolved in 10 µl of sterile distilled water. The DNA was eluted by incubating the tube at 45°C for 10 min.

Extraction of single-stranded phage DNA

The method used for isolation of single-stranded DNA used for nucleotide sequencing was that of Vieira and Messing (1987). Transvectants of DH5αF' with recombinant RF DNA from M13mp18 or M13mp19 were plated within a seeded agar overlay containing X-gal for blue/white colorimetric determination of recombinant phages. White plaques were first sized by agarose gel electrophoresis to identify the correct insert size within the recombinants. A single white plaque of the correct recombinant M13 phage was inoculated into a logarithmically growing culture of DH5αF' in 3 ml of 2xYT media and grown overnight at 37°C with vigorous shaking. The cells were then pelleted by centrifugation at 8000xg for 5 min. in a clinical table top centrifuge. 1.0 ml of the phage containing supernatant was recovered from the tube and transferred to an eppendorf centrifuge tube. 50 µl of

10 M ammonium acetate and 110 μ l of 40% w/v PEG-8000 were added and the tubes placed on ice for 20 min. The tubes were centrifuged for 15 min. (approx. 14,000xg) in an eppendorf centrifuge at 4°C. The precipitated phage particles were resuspended in 200 μ l of TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) and extracted twice with an equal volume of phenol (GIBCO-BRL) by gently mixing. The organic and aqueous phases were separated by centrifugation in an eppendorf centrifuge for 15 min. at 4°C. A final extraction was done with an equal volume of chloroform::isoamylalcohol (24:1). The remaining aqueous phase was transferred to a clean tube and the DNA precipitated by addition of 50 μ l of 10 M ammonium acetate and 600 μ l of prechilled absolute ethanol. This was placed at -70°C for 1 hr. The precipitated DNA was centrifuged and washed three times with 70% v/v ethanol as previously described for plasmid isolation. The total amount of ssDNA was dissolved in 20 μ l of sterile distilled water.

Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer-Mannheim, New England Biolabs, or Amersham. Single digests were accomplished by adding 1 μ l of purified DNA or 2-3 μ l of mini-prep DNA to a total reaction volume of 20 μ l containing the appropriate dilution of 10x restriction buffer provided by the manufacturer and 1-2 Units of enzyme. For

multiple digests involving enzymes with requirements for different ionic concentrations, the reaction was performed as above with the enzyme requiring the buffer of lower NaCl concentration, after which 4 μ l of the higher salt 10x buffer and enzyme added and the reaction volume increased to 40 μ l. Reactions were incubated at 37°C for 1 hr., mixed with an equal volume of loading dye (40% Ficoll; 10 mg/ml RNase A; 0.05% bromphenol blue) and analyzed by agarose gel electrophoresis.

Ligation of DNA for molecular cloning

Ligations of DNA were done by combining DNA fragments isolated from agarose gels with purified vector DNA or, in some cases, simply combining the desired fragments from the gel. The mixture of DNA fragments to be ligated was then bound to a silica matrix (GENECLEAN) and washed as described for isolation of DNA fragments. 7 μ l of the DNA resuspension was mixed with 2 μ l of 5x ligation buffer supplied by BRL and 1 Unit of T_4 DNA ligase added. The ligation mixture was incubated overnight at 12-14°C. 2 μ l of this was used per 100 μ l of competent cells for transformation.

Deletion of DNA utilizing exonuclease *Bal31*

Deletions of plasmid DNA were accomplished using exonuclease *Bal31* (BRL) of "slow" activity. Approximately 5 μ g of purified plasmid DNA was linearized with the appropriate restriction enzyme and ethanol precipitated as previously described. The DNA was washed three times in 70% ethanol as before and resuspended in 30 μ l of sterile distilled water. The DNA was then subjected to *Bal31* digestion by addition of 10 μ l of 5x *Bal31* buffer (100 mM Tris; 5 mM EDTA; 3 M NaCl; 60 mM CaCl_2 ; 60 mM MgCl_2 ; pH 8.1) and 10 Units of enzyme (1 Unit/ μ l). The reaction mixture was incubated at 20°C. It was estimated that approximately 12 to 15 bps were removed per second at this temperature. The time of exposure varied with the desired length of deletion. After the designated time, 10 μ l of 0.5 M EGTA was added to stop the reaction and the DNA precipitated with 15 μ l of 10 M ammonium acetate and 200 μ l of prechilled absolute ethanol. The DNA was again washed three times in 70% v/v ethanol and end-filled with DNA polymerase (Klenow fragment) in the presence of all four dNTPs. The DNA was then ligated as described before with synthetic oligonucleotide restriction site linkers. The modified DNA was digested with the appropriate restriction enzyme and religated in order to remove any tandemly inserted linkers. The DNA was used to transform *E. coli* as described below.

Transformation of *E. coli* and *K. oxytoca*

Transformation of bacterial cells was performed by a procedure similar to that of Cohen *et al.* (1972). Overnight *E. coli* cultures grown in either LB or 2xYT were diluted 1:50 into fresh media containing 10 mM MgCl₂ and grown in a Bellco Nephelo flask with vigorous shaking at 37°C until reaching approximately 75 Klett units (Klett-Summerson photoelectric colorimeter fitted with blue filter No. 42). This corresponded to a cell density of ca. 2×10^8 cfu/ml. The cells were placed on ice for 10 min. before collection by 8000xg for 5 min. in a clinical table top centrifuge. The cells were resuspended in 0.5 volumes of 75 mM CaCl₂ and placed on ice for 10 min. The cells were again centrifuged as before and resuspended in 0.1 volumes of 75 mM CaCl₂. The cells were usually placed at 4°C for 18-24 hrs. before transformation to increase competency (Dagert and Ehrlich, 1979). Approximately 50 ng of plasmid DNA was added to 100 μ l of competent cells in a 1.5 ml eppendorf tube and the mixture placed on ice for 10 min. The cells were then heat pulsed at 42°C for 2 min. and placed back on ice for 5 min. 1.0 ml of fresh LB broth was then added and the cells incubated for 60 min. at 37°C. Afterward, the cells were centrifuged for 10 sec. in an eppendorf centrifuge, resuspended in 20 μ l of LB broth, and plated onto selective media.

Analysis of cloned DNA by agarose gel electrophoresis

Fragments of restricted DNA were separated by agarose gel electrophoresis in a 1.0% agarose gel in TBE buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.0) run in a BIORAD horizontal unit. For the purpose of retrieving a DNA fragment from the gel, TBE buffer was replaced by TAE buffer (40 mM Tris-acetate; 2 mM EDTA; pH 8.0). Gels were typically electrophoresed at 20 V for 30 min. and then at 60 V for 2.5 hrs. The gel was stained with ethidium bromide and the DNA bands visualized by placing the gel on a Fotodyne Foto/prep I transilluminator. Gels were photographed using a Polaroid MP4 vertical camera fitted with a Kodak Wratten ultraviolet gelatin red filter No.18A using type 57 Polaroid film. The sizes of DNA fragments were determined by comparison to a 1 kb dsDNA ladder standard from BRL.

Bacterial Conjugation

Mating experiments were performed by two methods: a mini-filter method using a nitrocellulose filter and a plate spot method to test for the Kik phenotype. The quantitative filter membrane method was done by growing both donors and recipients overnight, diluting 1:10 into fresh liquid media, and growing the cells for 2 hrs. at 37°C to a density of 75 Klett units (approximately 2×10^8 cfu/ml). 90 μ l of donor culture was mixed with 10 μ l of recipient culture in an eppendorf centrifuge

tube and the entire mixture placed onto a 24 mm 0.45 μm pore size nitrocellulose filter disc resting on an LB agar surface prewarmed to 37°C. Mating on the filter was allowed to continue at 37°C for 2 hrs. The filter was then transferred to 2.0 ml of 85% saline and the cells washed from the filter by vortexing the tube for several minutes. The cell suspension was then diluted 10^{-4} in saline and 0.1 ml plated onto media selecting for the recipients. This method consistently gave a plate count of about 100 colonies when recipients alone were used as a control. Donors carrying plasmids to be tested for the Kik phenotype were easily scored for percent killing in comparison.

A qualitative test for the ability of donor cells to kill *K. oxytoca* was performed by growing donors to 75 Klett units as above and spotting 3 μl onto a lawn of recipient cells on minimal media. Zones of inhibition were scored on a three point scale system as +, ++, and +++, depending on the intensity of clearing within the spot. A zone of inhibition was scored as +/- if repeated spot tests gave inconsistent results between + and -. Originally, Rodriguez and Iyer (1981) described the killing of *K. oxytoca* by conjugative transfer of IncN plasmids. Killing mediated by plasmid pCU1 and other IncN plasmids was seen to occur with approximately 90-95% loss of viable cell count when plated onto minimal media. These plasmids were designated Kik⁺. Plasmids of incompatibility groups P and W were also seen to kill *Klebsiella*, but at a reduced rate of approximately 20% loss

of viable cell count. These plasmids were also designated Kik⁺. However, when defining regions of plasmid pCU1 required for killing, Thatte *et al.*, (1985b) described a derivative of pCU1 (pCU101) that had apparently lost killing function. Since this plasmid was found to kill *Klebsiella* at a reduced rate of 20% loss of viable cell count, it was designated, relatively, Kik⁻. The phenotype of plasmid pCU101 was thus used to define a region of pCU1 required for the Kik⁺ phenotype, *kikB*. Because of this discrepancy in nomenclature, any discussion of the phenomenon describing the killing of *K. oxytoca* warrants that the phenotypic terms Kik⁺ and Kik⁻ be more precisely defined.

Using the carefully controlled spot test described above (also see figure 19), I have developed a visual assay for the amount of killing seen within a spot mating. In this study, I use the following notations:

+++ denotes 90-95% loss of *K. oxytoca* viable cell count similar to that seen when the donor cell harbors plasmid pCU1.

++ denotes 50-89% loss of *K. oxytoca* viable cell count similar to that seen when the donor cell harbors plasmid pCU109.

+

denotes 20-49% loss of *K. oxytoca* viable cell count similar to that seen when the donor cell harbors plasmid pCU101.

+/- denotes 0-19% loss of *K. oxytoca* viable cell count indicating some variability.

- denotes complete lack of clearing zone

Also for clarity, I will use the term Kik⁺ only when the visual assay receives any mark of + or higher level of killing. That is, when killing approaches the level

of that seen with pCU101, it is deemed Kik^+ . *kikB* is thus defined as a region of pCU1 required to raise the level of killing from that of pCU101 to the level of pCU109.

Southern transfer and hybridization conditions

The transfer of DNA onto a solid matrix for detection of homologous sequences was a modified version of the blotting technique described by Southern (1975). Plasmid DNA was digested with various restriction enzymes and fragments separated by agarose gel electrophoresis in a 1.0% agarose gel in TBE buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.0). After the gel was stained in ethidium bromide and photographed, it was exposed to long wave UV irradiation by placing it on a transilluminator for 10 min. at high power. The gel was soaked in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 30 min. on a rotating platform. The gel was then placed in neutralization solution (0.5 M Tris; 1.5 M NaCl; 0.001 M EDTA; pH 7.2) for 30 min. A sheet of Hybond-N nylon filter (Amersham) was placed on top of the gel and a layer of 3MM Whatman paper was placed on top of the filter paper. A stack of paper towels held down with a 0.5 kg weight served to draw a pool of 20xSSC (3 M NaCl; 0.3 M sodium citrate) up through the gel and filter for 16-24 hrs. After transfer, the filter was removed and soaked in 2xSSC (0.3 M NaCl; 30 mM sodium citrate) to remove any adhering agarose and

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MICROCOPY RESOLUTION TEST CHART
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STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

allowed to air dry. Binding of the DNA to the filter was done by inverting the filter on a UV transilluminator for 5 min. at high power.

A radiolabelled probe of DNA was prepared by restriction of purified DNA and recovery of the fragment from the gel. Nick translation (Rigby *et al.*, 1977) was performed as described by Maniatis *et al.* (1982) using approximately 0.5-1 μ g of DNA and 25 μ Ci of radiolabelled deoxyadenosine [α - 32 P]-triphosphate (Amersham). The unincorporated nucleotides were separated from the radiolabelled DNA by passing it through two packed columns of Sephadex G-50. 500 μ l of 1 mg/ml salmon sperm DNA was added to the probe to act as a carrier. The probe DNA was denatured by boiling before adding it to a heat sealed bag containing the filter and hybridization solution.

Hybridization was done by a protocol supplied by Amersham. Prehybridizations and hybridizations were done in a final concentration of 6xSSC, 5xDenhardt's solution, and 0.5% SDS. Probe was added to the heat sealed bag after 2 hours of prehybridization at 65°C and the hybridization allowed to go 16-24 hrs. at 65°C. Post-hybridization washes were done as follows: two 15 min. washes in 2xSSC at 65°C, one 30 min. wash in 2xSSC/0.1% SDS at 65°C, and one 10 min. wash in 0.1xSSC at 65°C. After the washes, the filter was allowed to air dry. The filter was then placed inside a cassette with DuPont intensifying screens for exposure of Kodak X-OMAT AR film at -70°C overnight. The film was developed by an

AFP Imaging 14XL X-ray film processor following the procedure supplied by the manufacturer.

Nucleotide sequencing

Deoxynucleotide sequencing was done by using either modified T₇ DNA polymerase I (Sequenase) or Sequenase version 2.0 provided in a sequencing kit purchased from US Biochemical Corporation, Cleveland, Ohio, U.S.A. The method for sequencing was the dideoxy-termination technique described by Sanger *et al.*, (1977). The M13 universal primer was supplied with the Sequenase kit. Synthetic oligonucleotide primers specific for *kikA* were produced by Genetic Designs, Salt Lake City, Utah, U.S.A. and are described as follows:

Universal primer: 5'-GTAAAACGACGGCCAGT-3'

Tn5-1 primer: 5'-TAGGAGGTCACATGGAAGTC-3'

PNH-1 primer: 5'-ATAAAGCAGACTACCAGCTGC-3'

PNH-2 primer: 5'-AGTCTGCTTTATCTTCCTGCC-3'

PNH-3 primer: 5'-ACCCAATCACACGAAGGATGC-3'

PNH-4 primer: 5'-AGTCTGCTTTAGCTTCCTGCC-3'

PNH-5 primer: 5'-TTATGTGCATAGCGGGCAAGC-3'

PNH-6 primer: 5'-ACACGAAGGATCCTAGGAAGGC-3'

The sequencing reactions were done by annealing 0.5 pmol of primer to 4 µl of

purified ssDNA at 65°C for 10 min. and slowly cooling to 30°C. The primer was extended with radiolabelled deoxyadenosine [α -³⁵S]-triphosphate (Amersham) as recommended by the Sequenase protocol at 20°C for 5 min. The termination reactions were done at 37°C for 5 min. and the reactions halted by addition of STOP solution (95% formamide; 20 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF). Each reaction was denatured by boiling for 5 min. before loading 2-3 μ l onto a 6% polyacrylamide gel and electrophoresed at 30 mA for the desired length of time. The separation of DNA fractions was approximately 50 bases per hour. After electrophoresis, the gel was removed from the glass plates, placed in a fixative (10% v/v acetic acid; 10% v/v methanol) for 20 min., and dried under vacuum in a gel dryer. The gel was placed against Kodak X-OMAT AR film at 20°C overnight and later developed as described above.

Computer assisted DNA sequence analysis

Compilation, storage, and analysis of nucleotide sequences were performed using an IBM compatible "Microgenie" software package (Queen and Korn, 1984) purchased from Beckman Instruments Inc., Palo Alto, California, U.S.A. Prediction of open reading frames, translation products, and protein hydrophobicity profiles was made possible by software developed at California Institute of Technology (CALTECH) and supplied by the shareware program of the Molecular Biology Computer Research Resource (MBCRR) at the Dana Farber Cancer Institute, Boston, Massachusetts, U.S.A. DNA sequence comparisons against the GenBank data base were performed using the Genetic Computer Group (GCG) protocol over computerized electronic mail service. The identification of possible functional domains from protein sequences was done by comparison against a library of amino acid class covering (AACC) patterns for homologous families in the National Biomedical Research Foundation/Protein Identification Resource (NBRF/PIR) protein data base with the PLSearch algorithm (Smith and Smith, 1990) at the Dana Farber Cancer Institute, Boston, Massachusetts, U.S.A.

RESULTS - PART 1

Cloning and Characterization of *kikA*

Cloning of *kikA* onto a multicopy plasmid

Transposon mutagenesis of plasmid pCU1 has previously shown that three Tn5 insertions near the end of *tra* that is distal to *oriT* completely abolish the Kik phenotype (Kik⁻). These insertions were found to be closely clustered within an area extending from pCU1 coordinates 30.5 to 31.5 designated as *kikA* (see Figure 1). Cloning of *kikA* was accomplished by using a derivative of pCU1 with a transposon Tn5 insertion at pCU1 coordinate 32.4 (Thatte *et al.*, 1985a). This derivative, pCU171, was used first for construction of plasmid pCU701. pCU701 was created by digesting pCU171 with *Sma*I and religating (D. Denicourt, unpublished construct). A single *Sma*I site within Tn5 and one within pCU1 at coordinate 16.6 essentially allowed the removal of pCU1 DNA from coordinates 32.4 clockwise to 16.6, thus reducing the total size from 44.7 kb to 18.9 kb (Figure 1). pCU701 was then used as the source from which *kikA* was cloned. Digestion of pCU701 with *Hind*III and *Xho*I (a site for *Xho*I resides within Tn5) removed the fragment of DNA containing *kikA*. The *kikA* fragment (pCU1 coordinates 27.7 to 32.4) was retrieved from an agarose gel and ligated into the plasmid vector pACYC184 (Chang and Cohen, 1978) digested with *Hind*III and *Sal*I. The resulting plasmid, pCU1106 (L. Ailles, unpublished construct), was further reduced in size by digesting the plasmid with *Sal*I and religating. This removed the pCU1 DNA from coordinates 27.9 to 28.8 giving plasmid pCU1107. Figure 6 shows the construction of plasmids pCU1106 and pCU1107.

A test for pCU1107 to rescue three different Tn5 insertion mutants of pCU1 deficient in killing of *K. oxytoca* previously identified by Thatte *et al.* (1985a) was done by transforming plasmid DNA into each *E. coli* strain carrying pCU66, pCU167, or pCU184.

Figure 6. Construction of plasmid pCU1107 containing the *kikA* region. pCU701 is a *Sma*I deletion derivative of pCU171, a Tn5 insertion in pCU1 at 32.4 kb (Thatte *et al.*, 1985a). Arrows indicate restriction enzyme sites used in construction of PCU1106. Restriction of pCU1106 with *Sal*I and religation created plasmid pCU1107 (L. Ailles, unpublished construction). pCU1106 and pCU1107 are both maintained solely by the replication system of pACYC184.

Transformants of pCU1107 were then spot mated onto a lawn of *K. oxytoca* as described in materials and methods. In each case, killing was not observed (Kik⁻) indicating that the cloned fragment alone was not able to provide killing functions *in trans*.

IncN *oriT* is required *in cis* for killing of *K. oxytoca*

Rescue of the three *kikA::Tn5* mutants was accomplished by providing a functional *kikA* and IncN *oriT* *in cis*. Since plasmid pCU1107 was unable to rescue the mutations within *kikA*, it was possible that 1) *kikA* was nonfunctional when separated from pCU1, 2) a mutation had occurred within pCU701 to alter or delete *kikA*, or that *kikA* must be transferred during mating for the Kik phenotype. It was reasoned that the plasmid must be transferred efficiently to *K. oxytoca* for killing to occur since the killing phenomenon had been previously correlated with efficient transfer of pCU1 derivatives (Rodriguez and Iyer, 1981). Efficient transfer could have required that the *oriT* region be present on the plasmid carrying functional *kikA*. In order to provide IncN *oriT*, a mobile *oriT* cassette was created that could be retrieved by a variety of restriction enzymes. In so doing, the compatible restriction enzyme generated ends would be made such that the IncN *oriT* DNA fragment can be quickly and easily subcloned onto many different plasmid constructs. This proved to be very useful for later experiments designed to localize the *kikA* region.

The construction of a mobile IncN *oriT* cassette was accomplished as follows. A 3.8 kb *Bgl*II fragment of DNA from the *Bgl*II site of pCU1 coordinate 7.0 to a transposon Tn5 insertion at coordinate 9.3, excluding a region designated as *kikB*, was first cloned into pMK2004 (Kahn *et al.*, 1979) to create plasmid pSP1. In the course of precisely defining

oriT, deletions were created within pSP1 that did not affect the ability of this plasmid to be mobilized by the IncN transfer system. The resulting construct was pSP3. Further subcloning of a *Pst*I-*Bam*HI DNA fragment containing *oriT* into pUC13 (Vieira and Messing, 1982) created pUC13-P1 (S. Paterson, M.Sc. thesis, Carleton University, in preparation). The construction of pUC13-P1 provided *Eco*RI sites flanking a 1.5 kb DNA fragment that contained the *oriT* region of IncN plasmid pCU1. Cloning of this fragment into the unique *Eco*RI site of pUC1813 (Kay and McPherson, 1987) allowed the retrieval of *oriT* using the identical restriction sites provided by the multiple cloning sites of this vector. Figure 7 shows the construction of the resulting plasmid pUC1813-*NoriT*. DNA isolated from pUC1813-*NoriT* thus provides a mobile *oriT* cassette with several identical restriction enzyme sites on both ends without the further addition of linkers.

In order to clone IncN *oriT* onto the plasmid containing *kikA* described above, pUC1813-*NoriT* DNA was digested with *Hind*III to retrieve the 1.5 kb *oriT* fragment. The *oriT* fragment was ligated into the unique *Hind*III site of pCU1107 to create pCU1108. Digestion of pCU1108 with *Bam*HI and religation removed the *Eco*RI site clockwise from *oriT* so that the unique *Eco*RI site within the chloramphenicol resistance gene could be used for future mapping experiments. Figure 8 shows the construction of pCU1108 and pCU1110. Restriction analysis of pCU1110 revealed that in addition to removing the *Eco*RI site, a *Pst*I site in close proximity to *Eco*RI was also lost spontaneously upon ligation and subsequent transformation. This was found to be a common occurrence with cloning sites within the multiple cloning sites of pUC-derived plasmids (unpublished observations). Presumably, the event causing this occurs *in vivo*. Nevertheless, a unique *Bam*HI site

Figure 7. Construction of a mobile IncN *oriT* cassette. *oriT* can be easily retrieved from pUC1813-*NoriT* using a variety of restriction enzymes. Enzyme sites are H=*Hind*III; P=*Pst*I; X=*Xba*I; B=*Bam*HI; S=*Sma*I; K=*Kpn*I. Antibiotic markers Ap^r, Tc^r, and Km^r encode resistance to ampicillin, tetracycline and kanamycin, respectively. Plasmids pSP1, pSP3, pBP1, and pUC13-P1 containing the *oriT* region from pCU1 were constructed by S. Paterson (M. Sc. thesis, Carleton University, in preparation).

remained clockwise from *oriT* on pCU1110.

When either pCU1108 or pCU1110 is transformed into each *E. coli* strain carrying pCU66, pCU167, or pCU184 and transformants are used in spot matings on a lawn of *K. oxytoca*, recipient cells are killed (Kik^{+++}). This indicates that *kikA* must be mobilized into *K. oxytoca* for expression of the Kik phenotype and that the cloned *kikA* is potentially functional when present on plasmid pCU1107.

***kikA* resides within a 0.5 kb region**

Defining *kikA* within a 0.5 kb DNA fragment was accomplished by *Bal31* deletion analysis and providing IncN *oriT* in *cis*. Construction of plasmid pCU1110 showed that a functioning *kikA* region and *oriT* on the same plasmid could rescue Tn5 mutations within the *kikA* region of pCU1. Deletion analysis of pCU1110 proved to be problematic, however, for defining the area containing *kikA*. Since the cloning vector pACYC184 does not contain a convenient enzyme site between *EcoRI* and the *SalI* site (the *SalI* site was destroyed in the cloning of a *XhoI* end) for linearizing the *kikA/oriT* construct, it was necessary to create deletions within the plasmid not carrying *oriT*, pCU1107. In order to locate the end of *kikA*, pCU1107 was first linearized by digestion with *BglI*. Two *BglI* sites are located between the *SalI/XhoI* and the *EcoRI* site of pCU1107 (see Figure 8). *BglI* was chosen to linearize pCU1107 by cleaving it in a nonessential region (pCU1110 could not be used for such an experiment because it has an additional *BglI* site within the 1.5 kb *oriT* fragment not previously mapped). The linear DNA was then subjected to exonuclease *Bal31* as described in materials and methods. *XhoI* linkers were added, the DNA end-filled, and ligated. *E. coli*

Figure 8. Construction of pCU1110, a mobilizable plasmid containing *kik4* and the IncN *oriT* from plasmid pCU1. The restriction enzyme sites for *EcoRI*, *PstI*, and *HindIII* within the multiple cloning site of pUC1813 were spontaneously lost upon deletion of the smaller *Bam*HI fragment from pCU1108.

DH5 α transformants resistant to chloramphenicol were isolated and the plasmid DNA mapped by restriction enzymes *EcoRI* and *XhoI*. Selection for those plasmids with the chloramphenicol resistance gene intact limited the extent of deletion in the clockwise direction. Plasmid DNA from a number of clones was screened for loss of approximately 2.5 kb. Figure 9 shows the extent of deletions obtained in this manner.

In order to test these plasmids for a functional *kikA*, it was again necessary to provide *oriT* in cis. A select group of deletion derivatives was chosen and the *kikA* containing *EcoRI-SalI* fragment was subcloned in place of that on pCU1110. When tested for the Kik phenotype by spot test, each composite derivative gave a positive (Kik⁺⁺⁺) response indicating that the *kikA* region had not been deleted. To test for loss of *kikA* function from these plasmids, one of the derivatives (pCU1110-29) was further digested with *Bal31* after being linearized with *XhoI*. One deletion, pCU1110-29/21, extended into the *kikA* region and was negative (Kik⁻) by spot test. Figure 10 shows the extent of these deletions for defining the right end of *kikA* toward the transfer region. The least amount of DNA remaining that still conferred the Kik phenotype maps as a fragment of DNA 2.2 kb from the *SalI* site located at pCU1 coordinates 28.8 kb. The results here indicate that at least part of *kikA* resides between the terminal end of pCU1110-49 and that of pCU1110-29/21 (pCU1 coordinates 30.4 to 31.0).

Precise definition of the other end of *kikA* was done in a similar fashion. However, since it was desirable to eliminate extraneous DNA from the *kikA* containing plasmid, DNA fragments from two different deletion derivatives were combined to form a new construct with very little DNA between the *XhoI* site and the chloramphenicol resistance gene. pPH0

Figure 9. *Bal31* deletions of pCU1107. The solid lines indicate DNA from pCU1107 remaining after treatment with exonuclease *Bal31*. Each derivative obtained contains a *Xho*I restriction site linker at the point of deletion.

Figure 10. A partial map of plasmid pCU1 showing deletion analysis for defining the right end of *kikA*. The numbers indicate kb coordinates of pCU1. A section of the transfer region of pCU1 encoding the components for production of conjugative pili is shown. Five of the eight complementation groups (*traA* through *traE*) mapped within the transfer region are also shown. The solid black lines below the expanded region indicate DNA from pCU1 remaining on the plasmid derivatives. Thin lines indicate the DNA deleted from the constructs. An X marks the location of the *Xho*I linker. Open boxes indicate DNA from IS50 of transposon Tn5. The solid triangle indicates the insertion of Tn5 within pCU171, a Tn5 insertion in pCU1 at 32.4 kb (Thatte *et al.*, 1985a). The Kik phenotype for each plasmid is shown to the right.

was constructed by ligating the 3.1 kb *XhoI-HindIII* fragment of pCU1110-29 that contains the chloramphenicol resistance gene to the 3.8 kb *XhoI-HindIII* fragment of pCU1110-49 that contains *kikA* and *oriT*. Figure 11 shows a diagram of the construction of pPH0 and Figure 12 shows an agarose gel electrophoresis photograph of the three constructs described. Plasmid pPH0 was transformed into *E. coli* carrying either pCU66, pCU167, or pCU184 and tested for the Kik phenotype, it also exhibited a positive (Kik⁺⁺⁺) response.

Deletion analysis of pPH0 was performed by linearization of the DNA with *Bam*HI, treatment with exonuclease *Bal*31, end-filling, addition of *Bam*HI linkers, ligation, and transformation of *E. coli* DH5 α . Transformants were selected for chloramphenicol resistance. No attempt was made to select for those plasmids that were not deleted for *oriT*. DNA from several transformants was analyzed by restriction digests with *Bam*HI and *Xho*I. A select group of deletion derivatives were tested for *kikA* activity as before. All derivatives, including one that contained only 0.5 kb of DNA from *Bam*HI to *Xho*I (pPH0/1), restored the Kik⁺ phenotype to pCU66, pCU167, and pCU184 indicating that the entire *kikA* region is contained within 0.5 kb (pCU1 coordinates 30.5 to 31.0). Figure 13 shows deletions of plasmid pPH0 for defining the left end of *kikA*. Interestingly, none of the plasmids isolated in this way were devoid of *oriT*. Plasmid pPH0/1 was further analyzed by restriction enzyme digestion and shown to have only 200-300 bps of DNA left within the *oriT* segment.

Figure 11. Construction of plasmid pPH0. DNA fragments from two separate deletion derivatives of pCU1110, pCU1110-29 and pCU1110-49, were combined to create plasmid pPH0. Deletions of plasmid pPH0 were further used to define the left end of *kikA*. The *oriT* region of pCU1110, pCU1110-29, pCU1110-49 and pPH0 is from pCU1.

Figure 12. Agarose gel of DNA fragments used for construction of pPH0. *XhoI-HindIII* fragments from pCU1110-29 and pCU1110-49 were combined to form pPH0. Numbers to the left of the gel indicate the size of the DNA fragments in kilobases. The 3.0 kb *XhoI-HindIII* fragment of pCU1110-29 and the 3.8 kb *XhoI-HindIII* fragment of pCU1110-49 are indicated to the right. The total size of pPH0 is shown by linearization of the plasmid DNA with *XhoI* (also shown).

- Lane A: 1 kb DNA ladder
- Lane B: pCU1110-29 restricted with *XhoI+HindIII*
- Lane C: pCU1110-49 restricted with *XhoI+HindIII*
- Lane D: pPH0 restricted with *XhoI+HindIII*
- Lane E: pPH0 restricted with *EcoRI+HindIII*
- Lane F: pPH0 restricted with *XhoI*

Figure 13. A partial map of plasmid pCU1 showing deletion analysis for defining the left end of *kikA*. The numbers indicate kb coordinates of pCU1. A section of the transfer region of pCU1 encoding the components for production of conjugative pili is shown. Five of the eight complementation groups (*traA* through *traE*) mapped within the transfer region are also shown. The solid black lines below the expanded region indicate DNA from pCU1 remaining on the plasmid derivatives. Thin lines indicate the DNA deleted from the constructs. An X marks the location of the *Xho*I linker and a B marks the location of the *Bam*HI linker. The solid triangle indicates the insertion of Tn5 within pCU171, a Tn5 insertion in pCU1 at 32.4 kb (Thatte *et al.*, 1985a). The Kik phenotype for each plasmid is shown to the right. For the construction of pPH0, the parental plasmid from which the deletion plasmids were derived, see Figure 11.

***kikA* is conserved among IncN plasmids**

Plasmids of incompatibility groups P and W but not F and I share with IncN plasmids the ability to kill *K. oxytoca*. IncN, IncP, and IncW plasmids have in common efficient conjugative mechanisms and the ability to be transferred to and maintained in a wide variety of bacterial species. Thus they are considered broad host range plasmids. In addition, plasmids of these three incompatibility groups confer to the *E. coli* host sensitivity to the icosahedral bacteriophage PRD1. Since efficient genetic transfer via conjugation is required for Kik, this suggests that the mechanism by which they kill *K. oxytoca* and perhaps their mechanisms of conjugation to different species of bacteria may be related.

Plasmids RK2 (IncP) and pS-a (IncW) both exhibit some degree of killing (Rodriguez and Iyer, 1981). Therefore these plasmids were selected as models for studies concerning the Kik phenomenon. R144-*drd3* was included as a negative control since IncI plasmids do not kill *K. oxytoca*. Each plasmid was tested for the Kik phenotype by the spot method (see Figure 19). Table 5 provides a description of these plasmids and their Kik phenotypes. It is apparent from this table that the killing phenotype of each IncN plasmid was much higher than that of the IncP and IncW plasmids. Also, as expected, the IncI plasmid showed no killing of *K. oxytoca*. The results of the spot test correlate very well with the quantitative studies of these plasmids by Rodriguez and Iyer (1981).

Table 5. Plasmids tested for the Kik phenotype

Plasmid	Inc group	marker(s) ^a	Kik test (spot)	Kik test ^b (filter)	Source or Reference
pCU1	IncN	Ap,Sm,Sp	+++ 90		Konarska-Kozłowska and Iyer, 1981a
R46	IncN	Ap,Sm,Su, Tc	+++ 92		Datta, 1975
pKM101	IncN	Ap	+++ n.t.		McCann <i>et al.</i> , 1975
RK2	IncP	Ap,Km,Tc	+/-	12	Meyer, 1975
pS-a	IncW	Cm,Km,Sm, Su	+	20	Datta, 1975
R144drd3	IncI α	Km,Tc	-	0	Datta, 1975

^a Phenotypic designations indicate resistance to antibiotics, ampicillin (Ap), streptomycin (Sm), spectinomycin (Sp), sulfonamide (Su), tetracycline (Tc), kanamycin (Km), and chloramphenicol (Cm).

^b Results from Rodriguez and Iyer, 1981. Data is presented as percentage of *K. oxytoca* cells killed in quantitative filter matings. (n.t. = not tested).

The relationship of the killing observed with these different plasmids was explored by using DNA-DNA hybridization with the 0.5 kb *kikA* fragment isolated from IncN plasmid pCU1. Plasmid DNA was extracted from *E. coli* strains bearing three different IncN plasmids, pCU1, R46, and pKM101 that exhibit the Kik phenotype. Plasmids RK2 (IncP), pS-a (IncW), and R144-*drd3* (IncI α) were also extracted for hybridization experiments. pCU1, R46, pKM101, and R144-*drd3* DNA was restricted with *Hpa*I. RK2 was digested with a combination of *Eco*RI, *Bam*HI, and *Kpn*I in order to provide good separation of the DNA fragments. pS-a was digested with *Hind*III and *Pvu*I. The DNA fragments were electrophoresed through an agarose gel and transferred to a nylon membrane for hybridization. Figure 14 shows the results of the hybridization experiment. It can be seen that when the 0.5 kb *kikA* fragment is used as probe for DNA-DNA hybridization under the conditions described in Materials and Methods, *kikA* hybridizes to all three IncN plasmids but does not hybridize to any of the other incompatibility group plasmids tested. Therefore, *kikA* is conserved among these IncN plasmids but not within IncP and IncW plasmids known to kill *K. oxytoca*. The way in which these plasmids are able to kill *K. oxytoca* therefore does not appear to be the result of a gene homologous to *kikA* of the IncN plasmid pCU1. Since the experimental conditions were quite stringent, detection of dissimilar sequences was not possible. The possibility remains that a *kik* gene distantly related to *kikA* could be present on any of these plasmids.

Since all three IncN plasmids tested above were found to contain a region of homology to *kikA*, the possibility that the IncN plasmids have conserved *kikA* was further tested. The IncN plasmids tested by Rodriguez and Iyer (1981) all showed some degree of

Figure 14. Southern blot hybridization of *kikA* to different plasmids of incompatibility groups N, P, W and I. The IncI plasmid R144*drd3* was included as a negative control since this plasmid is known to be Kik⁻. The membrane filter was hybridized overnight with ³²P-labelled probe DNA as described in Materials and Methods. Sizing of the DNA bands was done by comparison to a 1kb DNA ladder run in an adjacent well. In some casses, multiple enzymes were used to obtain DNA fragments in the range of the standard size fragments. Numbers to the right indicate the size of the DNA fragments for the 1 kb ladder in kilobases. Numbers to the left indicate the size of the DNA fragment which hybridizes to *kikA*.

Lane	Plasmid	Inc group	Enzyme(s)
A	pCU1	N	<i>Hpa</i> I
B	R46	N	<i>Hpa</i> I
C	pKM101	N	<i>Hpa</i> I
D	RK2	P	<i>Kpn</i> I+ <i>Eco</i> RI+ <i>Bam</i> HI
E	pS-a	W	<i>Hind</i> III+ <i>Pvu</i> I
F	R144 <i>drd3</i>	I α	<i>Hpa</i> I

A B C D E F

8.5 →
6.5 →
4.8 →

≡ 5⁶⁷
-4
-3
-2
-1.6
-1

killing with only one exception, pTM558. The inability to detect killing by this plasmid has been correlated with its relatively low frequency of transfer to recipients during conjugation. It was therefore plausible that all the IncN plasmids have conserved *kikA*. To test this, plasmid DNA from a total of eight different IncN plasmids was hybridized with the 0.5 kb *kikA* region from pCU1 as probe. Seven of the plasmids tested were previously shown to be transfer efficient by Gill and Iyer (1982). The other, pKM101, has been extensively studied with regard to its conjugative transfer region (Winans and Walker, 1985a).

DNA from each of the IncN plasmids was restricted with *HpaI*, electrophoresed, transferred to a nylon membrane, and probed with *kikA*. Figure 15 shows the hybridization results and the size of the DNA fragment containing *kikA*. Each plasmid showed at least one fragment with homology to *kikA* indicating that *kikA* is conserved among the IncN plasmids. The presence of two bands for plasmid R46 may be the result of either two copies of the *kikA* gene or a *HpaI* site residing within *kikA*.

Figure 15. Southern blot hybridization of *kikA* to different plasmids of incompatibility group N. All plasmids were restricted with *HpaI*. The membrane filter was hybridized overnight with ³²P-labelled probe DNA as described in Materials and Methods. Sizing of the DNA bands was done by comparison to a 1kb DNA ladder run in an adjacent well. Numbers to the left indicate the size of the DNA fragments for the 1 kb ladder in kilobases. Numbers to the right indicate the size of the DNA fragment which hybridizes to *kikA*. The 4.8 kb fragment of pKM101 containing *kikA* is indicated. In shorter time exposures, two fragments of plasmid R46 were seen in the range of 6.5 kb.

	Size of <i>HpaI</i> fragment hybridizing to <i>kikA</i>
Lane A: R45	10.0 kb
Lane B: R46	6.5 kb
Lane C: R48	6.0 kb
Lane D: R113	11.0 kb
Lane E: R199	9.0 kb
Lane F: N3	15.0 kb
Lane G: pTM4245	6.5 kb
Lane H: pKM101	4.8 kb

Killing of *K. oxytoca* is independent of IncN *tra* and *oriT*

Evidence that the transfer region of pCU1 is not contributing to the Kik phenotype was presented by Rotheim *et al.* (1988). However, it could not be demonstrated that the *oriT* region of pCU1 does not play a role in the mechanism of killing by pCU1. To assert that the *kikA* region without any other sequence from pCU1 can display the Kik phenotype, the *oriT* region of a different plasmid, RK2 (that itself has no apparent homology to *kikA*) was used instead of *oriT* of pCU1 as a means of transferring *kikA* into *K. oxytoca*.

To create a plasmid containing only *kikA* and IncP *oriT* of RK2, it was first necessary to produce a DNA fragment containing *oriT* from the IncP plasmid RK2 with the correct restriction sites on the ends. Since it is inconvenient to repeatedly generate suitable ends by addition of synthetic linkers, a retrievable omega fragment containing the kanamycin resistance gene and *oriT* from RK2 (Selvaraj and Iyer, 1983) was cloned into pUC1318 (Kay and McPherson, 1987). The resulting construct, pNH-Kan/*oriT*, contains the 4.5 kb fragment of pGS39 (Selvaraj, 1983) conferring kanamycin resistance and *oriT* from RK2. The omega fragment can be retrieved using restriction enzymes *HindIII*, *XbaI*, *SmaI*, *KpnI*, and *EcoRI*. Figure 16 shows the construction of a mobilizable kanamycin resistance omega fragment containing the origin of transfer replication (*oriT*) from the broad host range plasmid RK2.

Two plasmids were constructed that contain *kikA* and IncP *oriT*. The 4.5 kb DNA fragment of pNH-Kan/*oriT* containing IncP *oriT* was retrieved using *HindIII* and ligated into the unique *HindIII* site of pCU1107. *E. coli* DH5 α transformants resistant to both chloramphenicol and kanamycin were then analyzed by restriction digests. Two classes of

Figure 16. Construction of a mobilizable kanamycin resistance omega fragment containing the origin of transfer replication (*oriT*) from the broad host range plasmid RK2 and the kanamycin resistance gene from Tn5. The Kan/*oriT* cassette containing the IncP *oriT* from RK2 can be easily retrieved from pNH-Kan/*oriT* using a variety of restriction enzymes. Enzyme sites are E=*EcoRI*; P=*PstI*; X=*XbaI*; B=*BamHI*; S=*SmaI*; K=*KpnI*.

recombinant clones were found. Class I were those in which the 4.5 kb fragment had inserted with the kanamycin resistance gene closest to the chloramphenicol resistance gene of pCU1107. Class II were those in which the 4.5 kb fragment had inserted in the opposite orientation with the kanamycin resistance gene closest to *kikA*. Orientation of the 4.5 kb fragment was determined by restriction with a combination of *Pst*I and *Eco*RI. A smaller 2.0 kb fragment in those of class I revealed the *Pst*I site within the kanamycin resistance gene to be closer to the *Eco*RI site of the chloramphenicol resistance gene of pCU1107. A construct was selected from those of class I and class II and were named pCU1139 and pCU1140, respectively. The construction of plasmids pCU1139 and pCU1140 is shown in Figure 17 and an agarose gel showing the restriction mapping of these two classes of derivatives is shown in Figure 18.

Plasmids pCU1139 and pCU1140 were transformed into *E. coli* carrying broad host range plasmid RP4 (IncP plasmids RP4 and RK2 have been shown to be identical by heteroduplex analysis by Burkardt, *et al.*, 1979). Each was then tested for killing of *K. oxytoca* by spot test. Plasmid pGS39 served as a control for a possible killing effect due to RK2 *oriT*, the kanamycin resistance gene from transposon Tn5, or the cloning vector pACYC184. As is seen in Figure 19, the presence of the pCU1139 or pCU1140, but not that of pGS39, increases the level of killing by RK2 from almost a negligible amount ($Kik^{+/-}$) to a killing frequency of pCU109 (Kik^{++}). This shows that providing *kikA* on a mobilizable plasmid with RP4 *in trans* allows efficient killing of *K. oxytoca*. Killing also occurs regardless of orientation of the 4.5 kb kan/*oriT* fragment. This indicates that mobilization of *kikA* by a mechanism independent of conjugative transfer mediated by

Figure 17. Construction of plasmids pCU1139 and pCU1140. The 4.5 kb Kan/*oriT* cassette from pNH-Kan/*oriT* was ligated into the unique *Hind*III site of plasmid pCU1107 to produce two classes of recombinant molecules. The *oriT* region on pCU1139 and pCU1140 is from RK2.

+

Figure 18. Schematic representation and restriction analysis of pCU1139 and pCU1140. Construction of two classes of recombinant plasmids was achieved by cloning the 4.5 kb Kan/*oriT* cassette from pNH-Kan/*oriT* into the unique *HindIII* site of plasmid pCU1107. Class I contains the Kan/*oriT* cassette with the kanamycin resistance gene (Km^r) closest to the chloramphenicol resistance gene (Cm^r). An agarose gel photograph of plasmid DNA restricted with *PstI* shows a banding pattern consistent with that expected for each class. Numbers to the right of the gel indicate the size of the DNA fragments in kilobases for the 1 kb ladder. Numbers to the left indicate the size of DNA fragments produced when each plasmid is restricted with *PstI*+*EcoRI*.

Lane 1: plasmid DNA restricted with *PstI*+*EcoRI*

Lane 2: plasmid DNA restricted with *BamHI*+*EcoRI*

Lane 3: 1 kb ladder.

CLASS I

CLASS II

Figure 19. Photograph of Kik spot test. *K. oxytoca* UN2979 was spread as a lawn of cells on minimal media supplemented with histidine as described in Materials and Methods. *E. coli* donors carrying plasmids were spotted onto the lawn. After 24 hours, the donors were scored for the Kik phenotype.

A) Test for killing by plasmids from various incompatibility groups:

Donor	Kik
J5/pCU1	+++
J5/R46	+++
J5/pKM101	+++
J5/RP4 (or RK2)	+/-
J5/pS-a	+
J5/R144 <i>drd3</i>	-

B) Test for killing by mobilization of pCU1139 or pCU1140 by the IncP conjugative broad host range plasmid RP4:

Donor	Kik
C600/pCU1	+++
J5/RP4	+/-
J5/RP4+pGS39	+/-
J5/RP4+pCU1139	++
J5/RP4+pCU1140	++

A)

B)

pCU1, can lead to the killing of *K. oxytoca*. The mechanism by which killing occurs does not require the specific *oriT* region from pCU1.

RESULTS - PART 2

Nucleotide sequence analysis and expression of *kikA*

Nucleotide sequence of the entire *kikA* region

The 0.5 kb fragment from pPH0 was isolated by restriction digestion with *Bam*HI and *Xho*I and cloned into M13mp18 and M13mp19 (Messing and Vieira, 1982) cut with *Bam*HI and *Sal*I. This was done in order to sequence both strands of *kikA* by reading from the universal primer. The sequencing was preformed by the Sanger method as described in Materials and Methods. After the the first round of sequencing, synthetic oligonucleotide primers were engineered in order to confirm the sequence for both strands of the DNA fragment. Figure 20 shows the strategy employed for sequencing the 0.5 kb *kikA* fragment. Figure 21 shows the nucleotide sequence of the *kikA* fragment.

Analysis of the *kikA* sequence revealed three putative open reading frames (ORF). ORF104 and ORF70 read in the direction from the *Xho*I site to the *Bam*HI site. ORF104 begins approximately 140 bps from the *Xho*I site and extends to 3 bps from the *Bam*HI site. ORF70 begins 62 bps into and is entirely contained within ORF104. These ORF's would be transcribed in the direction away from *traA* and the rest of the transfer region of pCU1 (anti-clockwise on the pCU1 map in Figure 1). ORF80 is in the other orientation (clockwise on pCU1). Figure 20 shows the salient features of the *kikA* fragment as determined by DNA sequencing. Putative promoter sequences were identified for ORF104 and ORF70 (indicated in Figure 21). Since no promoter sequence was seen for ORF80, it was not considered further. The ORFs and promoters will be further considered in the discussion section of this thesis.

Figure 20. Nucleotide sequencing strategy of the 0.5 kb *kikA* fragment. Sequencing employed bacteriophages M13-KA108 and M13-KA201 in order to confirm the sequence from both strands of DNA. Sequencing reactions were performed using either the universal primer designated as U or a number of engineered synthetic oligonucleotide primers designated PNH1-PNH6. The 0.5 kb fragment is shown as an open box. Open reading frames identified are shown below the *kikA* fragment. Fusion joints from two Tn5 insertions within *kikA* matched the sequence obtained and are shown by a circle and stem structure. Putative promoters P₁ and P₂ are shown as well as a possible ribosome binding site (rbs).

Figure 21. Nucleotide sequence of *kikA*. Open reading frames ORF104, ORF70, and ORF80 were identified. The start of each ORF is shown within the sequence by arrows. Putative *K. oxytoca* promoter P_1 (with corresponding -10 and -26 regions) and *E. coli* P_2 (with corresponding -10 and -35 regions) are shown as bold and underlined. A possible ribosome binding site (rbs) (Shine and Dalgarno, 1974) is also indicated (boxed). Relevant restriction enzyme sites are underlined. The fusion joints from two identical Tn5 insertions within *kikA* (pCU167 and pCU184) are shown by an asterisk.

Nucleotide sequence of *kikA*.

HindIII
AAGCTTGCATGCCTGCAGGTCGAGGTTTGTGTGTCGAATATATTGCAGGC

PstI
GATATAGGTTACAATCCATTATAACGCAGCGTTATTATTAAATATTACTT

-35 P₂ -10

ACCTTTATCAACATTCTGGCGAGTGTATATCTGAACAAACTGT TAAGGAC rbs

ORF104 *

AAAAAATGAAGAACTCTTAATACCTCTGATAGCAGCTGGTAGTCTGCT

ORF70

TTATCTTCCTGCCAGCCATGCTGAAGATCCCTGCAAAGTTATTATGTGCA

TGGCGGGCAAGCTCACCGGCGATAGCGGCGGAAGCGAGTGTAACAGT

GCTGAAGCTGCTTTCTTCAATATCGTTAAAAAGAACAAGCACGGCTTTT

TACCCAACCACACGAAGGATGCTAGGAAGGCTTTTCTTAATGAATGCCC

GATAATGGCGAAGGTGGAAGTAACCAGTCGATGATAAGCCAGATCATA

STOP (ORF70) ORF80

AGTAAATACGGGAAAGTTCGCTTATAGGCCGGATCCCGGGTACCGAGC

STOP (ORF104)

*** BamHI KpnI

TCGAATTC
EcoRI

Predicted proteins from ORF104 and ORF70

Identification of ORFs within the *kikA* fragment allowed for the prediction of the amino acid sequence of the putative *kikA* protein product(s). Figure 22 shows the predicted protein sequences from ORF104 and ORF70. As a first step in predicting a mechanism of killing, the predicted polypeptides from the ORFs were plotted for hydrophobic domains by the method of Kyte and Doolittle (1982). The hydropathic profiles are shown in Figure 23. The plot of ORF104 revealed a hydrophobic domain within the first 20 amino acids indicating a possible N-terminal signal sequence for translocation of the predicted protein. Two other hydrophobic domains were visible for ORF104, one spanning approximately 25-35 and 50-55 amino acids. A large hydrophilic portion was seen from amino acids 60-85. ORF70 showed one hydrophobic region spanning 20-30 and one hydrophilic region at 40-50 amino acids.

Figure 22. Amino acid sequences of predicted proteins from ORF104 and ORF70.

Predicted protein from ORF104.

MetLysLysLeuLeuIleProLeuIleAlaAlaGlySerLeuLeuTyrLeuProAlaSer
HisAlaGluAspProCysLysValIleMetCysMetAlaGlyLysLeuThrGlyAspSer
GlyGlySerGluCysAsnSerAlaGluAlaAlaPhePheAsnIleValLysLysAsnLys
HisGlyPheLeuProAsnHisThrLysAspAlaArgLysAlaPheLeuAsnGluCysPro
AspAsnGlyGluGlyGlySerAsnGlnSerMetIleSerGlnIleIleSerLysTyrGly
LysValArgLeu---

Predicted protein from ORF70.

MetLeuLysIleProAlaLysLeuLeuCysAlaTrpArgAlaSerSerProAlaIleAla
AlaGluAlaSerValThrValLeuLysLeuLeuSerSerIleSerLeuLysArgThrSer
ThrAlaPheTyrProThrThrArgArgMetLeuGlyArgLeuPheLeuMetAsnAlaArg
IleMetAlaLysValGluValThrSerArg---

Figure 23. Hydrophobicity profiles of putative translational products from ORF104 and ORF70. Hydropathic indexes for the predicted polypeptides from ORF104 and ORF70 within the 0.5 kb *kikA* fragment were calculated by the method of Kyte and Doolittle (1982) using a window of 11 amino acids. Hydrophobic domains are represented by a positive hydropathic index score.

Placement of *kikA* under the control of a strong *E. coli* promoter P_{lac}

In all experiments published thus far and all experiments described in this thesis, tests for the Kik phenotype have required that *E. coli* and *K. oxytoca* cells be brought together in a mating event. To determine if events during bacterial conjugation are required for killing, an attempt was made to establish *kikA* within *Klebsiella* while under control of the repressible *E. coli tac* promoter (de Boer *et al.*, 1983). The 0.5 kb *kikA* fragment was retrieved from M13-KA108 and M13-KA201 for cloning onto pJF118HE and pJF118EH (Fürste *et al.*, 1986) such that the ORFs would be in opposite orientations with respect to P_{lac} . Figure 24 shows the construction of plasmid pPH3 in which the strong *E. coli tac* promoter is positioned upstream of ORF80. Also shown is the construction of plasmid pPH4 in which the strong *E. coli tac* promoter is positioned upstream of ORF104 and ORF70. *K. oxytoca* UN2979 was transformed with each of these plasmids and tested for induction of Kik by addition of IPTG. First, various concentrations of IPTG were spotted onto a lawn of cells growing on minimal media. A clearing zone showed that IPTG could kill those cells carrying plasmid pPH4 but not those carrying pPH3. The level of IPTG required for killing was determined to be >0.1 M. Next, *K. oxytoca* carrying both constructs were grown to logarithmic phase and plated onto minimal media with and without 0.1 M IPTG. *K. oxytoca* UN2979 transformed with the vector DNA (pJF118HE) served as a negative control. The results of the induction experiment show that the presence of the P_{lac} vector pJF118HE has no influence on the viability of *K. oxytoca* in the presence of 0.1 M IPTG. With pPH3, where the *kikA* fragment is positioned such that ORF80 is driven by the *tac* promoter, no effect was seen on viable cell numbers. However, when plasmid pPH4 is established in

Figure 24. Construction of plasmids pPH3 and pPH4. Phage DNA from M13-KA201 provided the proper restriction enzyme sites for cloning *kikA* behind the *tac* promoter in opposite orientations. The *kikA* fragment was cloned onto pJF118EH and pJF118HE (Fürste *et al.*, 1986) to produce pPH3 and pPH4. P_{tac} is a hybrid of the -35 region of the *trp* promoter and the -10 region of the *lac* promoter (de Boer *et al.*, 1983). *lacI^q* is a constitutively expressed gene encoding the *lac* repressor (Farabaugh, 1978). *rrnB* is a transcriptional terminator from an *E. coli* ribosomal RNA operon (Brosius *et al.*, 1981).

K. oxytoca, a dramatic loss (>99.9%) of viable cell number is seen when plated onto media containing inducer. When *kikA* is in the opposite orientation, the level of killing is <5% (within plating error). These results clearly show that induction of *kikA* with IPTG gives a reduction of plating efficiency when *kikA* is in only one orientation (pPH4), that which provides ORF104 and ORF70 with the strong *tac* promoter. Figure 25 shows A) a photograph of the spot test for induction of *kikA* using different levels of IPTG and B) the percentage of viable cell counts (colony forming units) obtained from cultures containing the different plasmids. This indicates that the direction of transcription of *kikA* must be in the direction driving ORF104 and ORF70. This further provides strong evidence that the loss of viable cell count is directly related to the expression of *kikA* alone when resident *K. oxytoca*. When *E. coli* cells carrying pPH4 were tested for induction by IPTG, no loss in viability was detected.

Figure 25. Induction of *kikA* under control of the *tac* promoter.

A) Photograph of the induction of *kikA* by various levels of IPTG. 3 μ l of IPTG was spotted onto a lawn of *K. oxytoca* UN2979 carrying either pPH3 or pPH4. The results were scored as follows:

no IPTG	-
0.06 M IPTG	+
0.12 M IPTG	+
0.25 M IPTG	+++
0.5 M IPTG	+++
1.0 M IPTG	+++
2.0 M IPTG	+++

B) Percentage of viable cell count (VCC) when plasmids pPH3 and pPH4 are resident in *K. oxytoca* UN2979 with and without the presence of 0.1M IPTG in the plating media. UN2979 carrying the vector plasmid pJF118HE served as a negative control. Induction of *kikA* on plasmid pPH4 showed a decrease in plating efficiency (as colony forming units) indicating >99.9% of cells were unable to form colonies.

A)

B)

Tn5 insertions in *kikA* reside in the 0.5 kb fragment

Localization of three *kikA*::Tn5 mutations was accomplished by subcloning. Two Tn5 insertion mutants of *kikA*, pCU184 and pCU167 were further analyzed by nucleotide sequencing. DNA was extracted from *E. coli* strains containing each of these plasmids, restricted with *SalI*, and ligated into the *SalI* site of pUC19 (Yanisch-Perron *et al.*, 1985). *E. coli* DH5 α transformants were selected on media containing carbenicillin and X-gal. White colonies were then patched onto media containing kanamycin to test for the unselected marker of Tn5. Kanamycin resistant clones derived from each pCU1 derivative were then analyzed for their plasmid DNA by restriction digestion. These clones were designated as pUC19-66A, pUC19-167A, and pUC19-184A, to correspond to pCU66, pCU167, and pCU184 respectively. All pUC19-66 derivatives isolated showed a total *SalI* insert size of 10.6 kb. The fragment present therefore consists of pCU1 DNA from coordinates 39.0 counterclockwise to the internal *SalI* site of transposon Tn5. This shows that Tn5 had inserted with the kanamycin resistance gene closest to the transfer region of pCU1 in pCU66. pUC19-167A and pUC19-184A derivatives contained a *SalI* insert of 5.5 kb. These derivatives contained the pCU1 DNA from the internal *SalI* site of Tn5 counterclockwise to the *SalI* site located at 27.9. This indicated that the orientation of transposon Tn5 insertion in both pCU167 and pCU184 is opposite to that of pCU66, although these are Kik⁻. Figure 26 shows the regions of pCU1 cloned.

Curiously, all derivatives of this kind contained the extra *SalI* fragment from pCU1 coordinates 27.9 to 28.8. It therefore appears that this *SalI* fragment of pCU1 is indispensable for survival in *E. coli*, of plasmids containing the region of pCU1 from the

Tn5 insert within *kikA* to the *SalI* site at 28.8. This region is thus tentatively designated as *korA*. In addition, deletion of the smaller 300 bp *PstI* fragment within the *SalI-SalI* region of pCU1106 (pCU1 coordinates 28.5 to 28.8; see Figure 26) produced a plasmid exhibiting no signs of instability or further deletions. This deletion was possible either because *korA* was unaffected or part of a potential *kilA* region had been removed. A question mark identifies this area in Figure 26. *korA* could therefore extend from *SalI* at 27.9 to *PstI* at 28.8 (L. Ailles, unpublished results). The region spanning from *kikA* to the *PstI* site at 28.8 then contains a region designated as *kilA* that may extend as far as the *SalI* site at 27.9. The observation that varying lengths of deletions were associated with attempts to clone from a Tn5*lac* insertion (pNH142) at 30.0 counterclockwise to the *PstI* site at 28.8 supports this conclusion (data not shown).

Evidence for an additional region designated *kilB* on pCU1 is the inability to construct plasmid derivatives containing a fragment of DNA from Tn5*lac* insertion at pCU1 coordinate 30.0 (pNH142) to the *KpnI* site of pCU1 at 34.0. The ability to clone from Tn5 insertion 171 to the *SalI* site at 28.8 excludes *kilB* from this fragment. Thus, the *kilB* region is located between 32.4 and 34.0 on pCU1. The derivative pUC19-66A containing pCU1 DNA from *kikA* to the *SalI* site at 39.0 must therefore encode the override gene *korB* located between the *KpnI* site at 34.0 and the *SalI* site at 39.0.

The pUC19 derivatives above were constructed in order to sequence the fusion joints of each Tn5 insertion within *kikA*. The 4.0 kb *EcoRI-HindIII* fragment of pUC19-167A and pUC19-184A were subcloned into M13mp18 and sequenced using a primer designed for the end of Tn5 (primer Tn5-1). The point of insertion for each was found to

Figure 26. 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 Tn5lac 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 Tn5lac 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*.

be identical and matched that of the *kikA* sequence previously shown. The insertion point of Tn5 in pCU167 and pCU184 is shown in Figure 21. This provides strong physical evidence that the 0.5 kb fragment of pPH0 does in fact contain the *kikA* region. Several attempts at subcloning the DNA fragment from the end of Tn5 to the *KpnI* site at 34.0 of pCU1 from pUC19-66A failed. Presumably, this was due to exclusion of *korB*.

An attempt to create an *in vivo* transcriptional fusion of *lacZ* to *kikA*

Three important observations prompted an attempt to obtain *lac* fusions to *kikA*:

1) killing was only observed when *K. oxytoca* cells were used as recipients in mating experiments, whereas *E. coli* recipients did not show a reduction in viable number, 2) conclusions drawn from earlier cloning experiments suggested that *kikA* is the main factor contributing to the Kik⁺ phenotype, and 3) earlier work showed that *kikB* was contributing to the efficient killing of *K. oxytoca*.

The possibility that *kikA* was under control mechanisms was explored using Tn5*lac*, a transposable transcriptional fusion vehicle (Kroos and Kaiser, 1984). C600/pCU109 was transformed with ColE1::Tn5*lac* DNA and plated onto media containing both chloramphenicol and kanamycin. More than 1000 transformants were then pooled together and growth to log phase for mating with C600 Nal^r. Transconjugants resistant to all three antibiotics were then pooled again for a second round of mating with C600 Rif^r. This was done in order to eliminate those insertions that occurred within the transfer region as these would also test negative for killing.

Upon testing each derivative for killing by spot test, 20 possible insertions were identified as Kik⁻. Plasmid DNA was extracted from each derivative and the site of insertion mapped by restriction digestion with *Kpn*I and *Bam*HI. The 20 derivatives were separated into two classes based on the restriction fragment pattern. Class I showed an identical restriction pattern that mapped Tn5*lac* 9.5 kb clockwise from the *Bam*HI site within pCU109. This placed the transposon at pCU1 coordinate 30.0 to 30.5 near the *kikA* region and in the clockwise orientation. Since class I derivatives were identical, they were most

likely siblings of the same insertion derivative. One of these was named pNH142 and selected for further study. pNH142 exhibits a reduced Kik phenotype (+) comparable to that of pCU101. It was therefore of interest. Plasmids of Class II showed a pattern similar to that of pCU109 without Tn5*lac* insertion.

Plasmids pCU109 and pNH142 were tested for β -galactosidase activity by mating C600 donors with *E. coli* DH5 α or *K. oxytoca* UN2979 recipients. Transconjugants were selected by their ability to grow on minimal media supplemented with histidine and containing chloramphenicol and X-gal. Surprisingly, colonies arising from either DH5 α /pNH142 or UN2979/pNH142 were blue on X-gal indicating that Tn5*lac* had inserted downstream from a promoter that is active in both *E. coli* and *K. oxytoca*. Controls containing pCU109 produced white colonies on X-gal. Since it was possible that Tn5*lac* in pNH142 had fused the *kikA* promoter to the *E. coli* gene for β -galactosidase, this insertion derivative was used for further study. A subclone of the leftward portion of the Tn5*lac* insertion was constructed and the fusion joint sequenced. The sequence of the fusion point of Tn5*lac* did not match that of the entire *kikA* region determined by other means. Therefore it was concluded that pNH142 was a *lacZ* fusion to a promoter proximal to *kikA* and not within *kikA*.

DISCUSSION

Discussion

It has been observed that only a small fraction (5 to 10%) of cells survive after plasmids of the incompatibility group N are transferred from *E. coli* donors to *K. oxytoca* recipients. This phenomenon, designated as Kik (*k*illing of *K*lebsiella), requires efficient transfer of plasmid DNA and is shared by all conjugative plasmids of incompatibility group N tested and to a lesser degree by IncP and IncW group plasmids (Rodriguez and Iyer, 1981). It has also been shown that Kik is not mediated by any substance that diffuses extracellularly from *E. coli* to *K. oxytoca* (Gill and Iyer, 1982). Specific interest in the IncN plasmid pCU1 is due not only to its extremely efficient system of conjugation between strains of *E. coli*, but also to its ability to be transferred and maintained in a wide variety of Gram-negative bacteria (Krishnan and Iyer, 1988). Plasmid pCU1 has been the subject of several investigations concerning its transfer region (Thatte and Iyer, 1983; Thatte *et al.*, 1985a) and its broad host range replication properties (Kozłowska *et al.*, 1987; Krishnan and Iyer, 1990).

An interesting aspect of broad host range plasmids is their stability within differing genetic backgrounds. Studies of the broad host range plasmid RK2 have revealed a highly complex system of regulatory elements involved in plasmid replication and stability (reviewed by Thomas and Helinski, 1989). The specific killing of *K. oxytoca* thus presented itself as an opportunity to explore the possibility

of host-dependent gene expression of a broad host range plasmid other than RK2. Previous studies of the transfer region of pCU1 have provided evidence that two regions, *kikA* and *kikB*, are involved in the Kik phenotype (Thatte *et al.*, 1985a; Thatte *et al.*, 1985b). As an initial step in exploring the basis of killing, this study was concerned with the isolation and molecular characterization of the *kikA* region from pCU1.

Three transposon Tn5 mutations in proximity to the transfer region of pCU1 defined the location of *kikA* (Thatte *et al.*, 1985a). These Kik⁻ mutations enabled us to define the *kikA* region of pCU1 and map its precise location to within a 0.5 kb fragment. When this DNA was used as a hybridization probe, homology to *kikA* was detected with all other IncN group plasmids tested. This provided molecular evidence that the *kikA* region is conserved in this group of plasmids. In the closely related plasmid, pKM101 (McCann *et al.*, 1975), and its parental plasmid R46 (Langer and Walker, 1981; Brown and Willetts, 1981), the hybridization results indicated that the *kikA*-like region is in an analogous position to that of pCU1.

When the *kikA* DNA was used to probe broad host range plasmids of incompatibility groups P and W, no homology was detected under the conditions employed. This indicated that either 1) the killing of *K. oxytoca* observed with these plasmids was not the result of a *kikA*-like gene, or 2) such a gene exists but is sufficiently diverged that the stringent hybridization conditions used limited its

detection. This leaves open the possibility that such a gene does exist on these plasmids, but is quite distantly related to *kikA*. Further studies of these regions should provide clues as to why the killing phenomenon is so widely conserved in plasmids of broad host range.

In all studies of the Kik phenomenon, it was necessary to provide a conjugatively proficient plasmid within *E. coli* donors to observe killing. This led to the notion that all or part of the pCU1 transfer region was involved in the mechanism of killing. It was also possible that events occurring during or shortly after the mating event caused killing. This implied that the transfer process itself was providing the means of killing. Recently, Rotheim *et al.* (1988) have eliminated a role of the components of the transfer region in killing by providing the transfer region of pCU1 on a non-mobilizable plasmid. Transfer of plasmids, each containing a Tn5 mutation in a different complementation group, still produced a Kik⁺ phenotype. The possibility of either *oriT* or the mating event itself was then left open. We considered the specific role of the IncN *oriT* in the killing of *K. oxytoca* by providing the heterologous *oriT* region from the IncP plasmid RK2. Transfer functions, provided by a helper plasmid RP4, mobilized a plasmid construct containing the *kikA* gene and the RK2 *oriT*. Here, the Kik phenotype was seen at a level as if the *oriT* of pCU1 were used to mobilize the *kikA* plasmid, thus showing that the IncN *oriT* region is not specifically required for killing. Furthermore,

evidence that the conjugative process itself is not necessary to produce the Kik phenotype was provided by construction of a plasmid in which the transcription of *kikA* was under control of the repressible *tac* promoter. Induction of *kikA* by IPTG showed a dramatic decrease in viable cell numbers, showing that killing can occur within *K. oxytoca* and independantly of an interaction with *oriT* or the process of conjugation. Another important observation is that *kikA* does not require the additional function of any other regions within pCU1 for the lethal effect.

As a contribution to the understanding of the killing mechanism, we further analyzed the *kikA* region by nucleotide sequencing. The DNA sequence of *kikA* was compared to those genes involved in the *kill/kor* regulon of RK2 (reviewed by Thomas and Helinski, 1989). No significant homology was found between the *kikA* DNA sequence and any sequenced *kil* gene of RK2. This was not surprising since we did not detect hybridization of the *kikA* probe to RK2 DNA. A comparison of the DNA sequence of *kikA* to the coupled cell division genes *ccdB* (Miki *et al.*, 1984), *kid* (Bravo *et al.*, 1988), and *pemK* (Tsuchimoto and Ohtsubo, 1989) showed no significant homology. Also, no significant homology was found between *kikA* and *hok* (Gerdes and Molin, 1986) or any of the *hok*-related genes including *flmA* (Golub and Panzer, 1988), *smB* (Akimoto *et al.*, 1986), and *pnd* (Ono *et al.*, 1987). We compared the DNA sequence of *kikA* to the sequenced genes encoding microcin B17 (Genilloud *et al.*, 1989) and found no significant homology. In order

to examine whether any similarities exist between *kikA* and any other known genes, the DNA sequence was used to search a "Microgenie" DNA sequence database and the GCG Genbank DNA sequence data base for DNA sequence homology. The results obtained showed that no other known or sequenced gene contains significant homology to *kikA*. Thus *kikA* appears to be unique within those plasmid genes which are potentially lethal to the host.

The DNA sequence of the *kikA* region revealed three putative open reading frames: ORF104, ORF70, and ORF80 (see Figures 20 and 21). Inspection of ORF80 shows that only 31 bps exist between the *Bam*HI linker of pPH0/1 (Figure 13) and the putative ATG start codon. Since the complementation tests for *kikA* show that the minimal *kikA* DNA fragment must contain its own promoter and no consensus promoter sequence is present, ORF80 most likely is not involved in the Kik phenotype. In addition, we cloned the *kikA* region in both orientations behind the inducible *tac* promoter on pJF118HE and showed that *K. oxytoca* cells carrying only the plasmid derivative in which ORF104 and ORF70 would be transcribed were killed when induced with IPTG.

A detailed examination of the *kikA* sequence leading into ORF104 and ORF70 shows a possible *E. coli* σ^{70} promoter consensus sequence upstream from both ORF104 and ORF70 designated P₂. This promoter has a distance between an atypical -35 region of GTTACA and a -10 region of TATTAT of 21 bps. The

optimal distance is 17 bps for recognition by *E. coli* RNA polymerase (Hoopes and McClure, 1987). This would indicate that if this is the promoter utilized for expression of *kikA* in *E. coli*, it would most likely be one of low promoter strength (Hawley and McClure, 1983). Another candidate for a *kikA* promoter is a *K. pneumoniae* σ^{60} consensus sequence of a -26 region of CTGGC and a -14 region of TATCT before the start codon of ORF104 designated as P_1 . The consensus sequence is a -26 region of CTGGC and a -14 region of TTGCA. Promoters of this type are frequently recognized under conditions imparting global regulation of a number of genes such as nitrogen starvation (Hoopes and McClure, 1987; Beynon *et al.*, 1983).

Two transposon Tn5 insertions within *kikA* that were Kik^- were analyzed by nucleotide sequencing. The fusion joints for these insertions were found to be identical and positioned such that Tn5 disrupts ORF104 but not ORF70. However, the insertions also separate ORF70 from both of the possible promoters identified, effectively preventing transcription of ORF70. Therefore we could not exclude either ORF from being involved in *Kik*. It must also be kept in mind that killing may involve a combination of products from ORF104 and ORF70 or an RNA molecule.

To understand the mechanism of killing, we considered two possible roles by which a potentially lethal gene was able to kill *K. oxytoca* but not *E. coli* cells: 1) expression of *kikA* could be transient or cell specific for *K. oxytoca*, and 2)

expression of *kikA* could occur in both *E. coli* and *K. oxytoca* but that *E. coli* cells are not susceptible to the lethal effect. Since we identified a possible promoter that could function specifically in *K. oxytoca* and another that would be utilized in *E. coli* and *K. oxytoca*, we tested *E. coli* cells for susceptibility to the *kikA* product by providing the strong *E. coli tac* promoter upstream of ORF104 and ORF70. When expression of *kikA* was induced, no loss of viability was observed for *E. coli*. Assuming that the *kikA* product is equally stable in *E. coli* and *K. oxytoca*, we concluded that the *kikA* product is specifically lethal to *K. oxytoca*. The cell specific expression of *kikA* from the promoter P₂ therefore seems quite unlikely.

The mechanism by which *kikA* causes the killing of *K. oxytoca* cells is an interesting topic, however, any mention of possible ways in which the lethal event may occur is speculative at this point. As a method of predicting a possible mechanism of killing, we examined the putative translational products from ORF104 and ORF70 more closely. A first step in identifying which ORF may be killing *K. oxytoca* was a prediction of hydrophilic domains for each. Interestingly, ORF104 contains a hydrophobic region within the first 20 amino acids that showed general features of a typical bacterial signal peptide (Pugsley, 1989) and comparison of the possible signal sequence to those proteins known to be transported to the outer membrane (Sjöström *et al.*, 1987; Nikaido and Wu, 1984) showed a significant similarity. Since other lethal genes such as those of the *hok*-related family and some

of the colicins act by perturbing the cellular membrane (Gerdes, 1990; Hardy, 1975), we considered the product from ORF104 to be a possible candidate for a killing protein. It was also taken into consideration that all three Tn5 mutations in *kikA* were not only Kik⁻ but also conferred to *E. coli* resistance to the phage PRD1. The Kik⁺ and PRD1^s phenotypes have yet to be distinguished. It is possible that the two phenotypes can be separated by mutation. If they can be, then perhaps more attention should be given to the predicted peptide from ORF70. No mutations have yet been identified which produce a Kik⁻ PRD1^s phenotype.

As an attempt to predict the activity of the predicted protein from ORF104, a computer assisted search of the amino acid sequence was compared against a "Microgenie" protein data bank. No significant matches to proteins of known function were found. Another approach was to search a database for a possible match to AACC patterns (Smith and Smith, 1990). The amino acid sequence was compared to a consensus pattern library using the PLSearch algorithm as described in Materials and Methods. The results showed that this protein did not significantly match with any consensus patterns in the library.

Since *E. coli* cells are not sensitive to the effect of *kikA*, it can be predicted that a target within *K. oxytoca* is available for the lethal event. There is little doubt that this target is involved in an essential process for viability of *K. oxytoca* cells. Future experiments may be directed at the target on which the *kikA* product acts

within *K. oxytoca* and we predict that it should be possible to isolate mutants of *K. oxytoca* that are resistant to killing. We believe that the lethal event is either circumvented or checked in *E. coli*. For example, it is likely that the cellular process that acts as the target is sufficiently different in *E. coli*. *E. coli* cells could also provide an override gene product that blocks the action of *kikA*.

A role for the *kikA* in plasmid inheritance seems paradoxical. Other known lethal genes prohibit loss of the plasmid by killing those that do not retain it. We do not know why *kikA* kills cells which receive pCU1 during mating or why *kikA* is so highly conserved other than providing an ecological advantage for reducing the number of competing bacteria.

Study of *kikA* has been hampered by difficulties in cloning specific restriction fragments of pCU1. When these regions were cloned onto a high copy number plasmid vector, spontaneous deletions of DNA were often observed. Preliminary evidence presented here suggests that at least two *kill/kor* systems may be operating within pCU1. Several attempts at cloning particular DNA fragments from pCU1 have indicated that at least one *kor* gene resides very close to or overlapping with the *kikA* gene. In the course of studying the *kikA* region, we have tentatively mapped two *kill/kor* systems on pCU1 (see Figure 26).

Thatte *et al.* (1985a) have attempted to saturate the entire transfer region of pCU1 with transposon Tn5. In doing so, two regions of DNA were identified in

which no Tn5 insertions were mapped. One of these regions is located between complementation groups *traC* and *traD*. It is interesting that the location of *korB* mapped on pCU1 corresponds with this region of the pCU1 transfer region. It is possible that this region prohibits Tn5 mutations because disruption of *korB* would be detrimental to plasmid stability.

In pKM101, two *kil* genes and two *kor* genes have been mapped close to or within the transfer region. The *kil* regions have been found not to determine the Kik phenotype of pKM101 because Tn5 mutations in *kilA* and *kilB* were not deficient in killing *K. oxytoca* (Winans and Walker, 1985c). The location of a *kikA* homologous region on pKM101 corresponds with the location of *kilA* and *korB*. If *kilA* regions on both pCU1 and pKM101 are in the same location, the *kikA* region specified by pKM101 would map between the *HpaI*-1 site and *kilA*. Tn5 mutations within pGW1584, a derivative of pKM101 containing this region, give rise to a low number of *E. coli* transformants which are slow-growing. In addition, plasmid DNA from these transformants was recovered in extremely low quantities. Winans and Walker (1985c) refer to this as the Sic phenotype and suggest that it is due to a low level of *kilA* expression caused by transcriptional polarity of Tn5 insertions. Although the Tn5 insertion in *kilA* of pKM101 was tested for the Kik phenotype, to my knowledge none of the mutations that produce the Sic phenotype were tested for Kik. Since the direction of transcription for *kikA* appears to be the same for

kilA of pKM101, it is possible that these insertions are within the *kikA* region and that *kikA* and *kilA* lie within the same transcriptional unit.

Although the mapping of *kil/kor* regions on pCU1 at this time is preliminary at best, there is little doubt pCU1 and pKM101 are sufficiently different to exclude *kil/kor* functions from pCU1. It is hoped that this information will set the stage for future study of *kil/kor* systems in pCU1. An intensive study of these regions may provide an explanation for some of the aberrant results observed when fragments of pCU1 are cloned onto high copy number plasmid vectors.

Information concerning the possible role of *kikB* is unclear and contradictory. Study of the *kikB* region showed that providing a cloned *kikB* from either pCU1 or pCU109 did not restore the reduced Kik phenotype of pCU101 to the level of that of pCU109 while *in trans*. This is not in agreement with previous results of Gill (1985). A possible reason for the reduction in killing observed when this plasmid is conjugatively transferred to *K. oxytoca* could be that 1) the plasmid is transferred at a frequency lower than that of pCU109, or 2) the plasmid is unstable and is lost from either *E. coli* donor cells in absence of selection or *K. oxytoca* recipients shortly after transfer. The region of pKM101 corresponding to *kikB* of pCU1 has been shown to contain a region that provides plasmid stability under specific conditions. The removal of the region (*stb*) leads to loss of the plasmid (Winans and Walker, 1985a).

The increase in killing observed when the *kikB* region is present may be due to an involvement of this region by 1) enhancement of expression of *kikA* or 2) a specific interaction between products of both *kikA* and *kikB*. Study of the *kikB* has been delayed by the inability to isolate mutations of this region. Of 33 Tn5 insertions mapped to the *kikB* region, none were found to affect the Kik phenotype. The reason for this was accommodated by suggestion that *kikB* may only be a very small segment of DNA (Thatte *et al.*, 1985a). Thus far, no mutations of *kikB* have been isolated.

As an attempt to explore a possible role of *kikB*, we sought to create a transcriptional fusion of *kikA* to *lacZ* and measure the level of gene expression using an indicator molecule. In all attempts, we failed to obtain the desired fusion. This may be due to the relatively small size of the *kikA* region in comparison to pCU1 or that the *kikA* gene product is translocated to the outer membrane, fusions of which would be lethal. Future study of the *kikB* region may be more productive using a reporter molecule more amenable to transport such as the periplasmic protein alkaline phosphatase.

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A resolution test chart featuring various patterns of horizontal and vertical lines. The patterns are arranged in a grid-like fashion, with some patterns being larger and more prominent than others. Numerical values are placed next to the patterns, indicating the resolution or frequency of the lines. The values include 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.6, 6.3, 7.1, 8.0, 9.0, 10.0, 11.2, 12.5, 14.0, 16.0, 18.0, 20.0, 22.5, 25.0, 28.0, 32.0, 36.0, 40.0, 45.0, 50.0, 56.0, 63.0, 71.0, 80.0, 90.0, 100.0, 112.0, 125.0, 140.0, 160.0, 180.0, 200.0, 225.0, 250.0, 280.0, 320.0, 360.0, 400.0, 450.0, 500.0, 560.0, 630.0, 710.0, 800.0, 900.0, 1000.0, 1120.0, 1250.0, 1400.0, 1600.0, 1800.0, 2000.0, 2250.0, 2500.0, 2800.0, 3200.0, 3600.0, 4000.0, 4500.0, 5000.0, 5600.0, 6300.0, 7100.0, 8000.0, 9000.0, 10000.0, 11200.0, 12500.0, 14000.0, 16000.0, 18000.0, 20000.0, 22500.0, 25000.0, 28000.0, 32000.0, 36000.0, 40000.0, 45000.0, 50000.0, 56000.0, 63000.0, 71000.0, 80000.0, 90000.0, 100000.0, 112000.0, 125000.0, 140000.0, 160000.0, 180000.0, 200000.0, 225000.0, 250000.0, 280000.0, 320000.0, 360000.0, 400000.0, 450000.0, 500000.0, 560000.0, 630000.0, 710000.0, 800000.0, 900000.0, 1000000.0, 1120000.0, 1250000.0, 1400000.0, 1600000.0, 1800000.0, 2000000.0, 2250000.0, 2500000.0, 2800000.0, 3200000.0, 3600000.0, 4000000.0, 4500000.0, 5000000.0, 5600000.0, 6300000.0, 7100000.0, 8000000.0, 9000000.0, 10000000.0, 11200000.0, 12500000.0, 14000000.0, 16000000.0, 18000000.0, 20000000.0, 22500000.0, 25000000.0, 28000000.0, 32000000.0, 36000000.0, 40000000.0, 45000000.0, 50000000.0, 56000000.0, 63000000.0, 71000000.0, 80000000.0, 90000000.0, 100000000.0, 112000000.0, 125000000.0, 140000000.0, 160000000.0, 180000000.0, 200000000.0, 225000000.0, 250000000.0, 280000000.0, 320000000.0, 360000000.0, 400000000.0, 450000000.0, 500000000.0, 560000000.0, 630000000.0, 710000000.0, 800000000.0, 900000000.0, 1000000000.0, 1120000000.0, 1250000000.0, 1400000000.0, 1600000000.0, 1800000000.0, 2000000000.0, 2250000000.0, 2500000000.0, 2800000000.0, 3200000000.0, 3600000000.0, 4000000000.0, 4500000000.0, 5000000000.0, 5600000000.0, 6300000000.0, 7100000000.0, 8000000000.0, 9000000000.0, 10000000000.0, 11200000000.0, 12500000000.0, 14000000000.0, 16000000000.0, 18000000000.0, 20000000000.0, 22500000000.0, 25000000000.0, 28000000000.0, 32000000000.0, 36000000000.0, 40000000000.0, 45000000000.0, 50000000000.0, 56000000000.0, 63000000000.0, 71000000000.0, 80000000000.0, 90000000000.0, 100000000000.0, 112000000000.0, 125000000000.0, 140000000000.0, 160000000000.0, 180000000000.0, 200000000000.0, 225000000000.0, 250000000000.0, 280000000000.0, 320000000000.0, 360000000000.0, 400000000000.0, 450000000000.0, 500000000000.0, 560000000000.0, 630000000000.0, 710000000000.0, 800000000000.0, 900000000000.0, 1000000000000.0, 1120000000000.0, 1250000000000.0, 1400000000000.0, 1600000000000.0, 1800000000000.0, 2000000000000.0, 2250000000000.0, 2500000000000.0, 2800000000000.0, 3200000000000.0, 3600000000000.0, 4000000000000.0, 4500000000000.0, 5000000000000.0, 5600000000000.0, 6300000000000.0, 7100000000000.0, 8000000000000.0, 9000000000000.0, 10000000000000.0, 11200000000000.0, 12500000000000.0, 14000000000000.0, 16000000000000.0, 18000000000000.0, 20000000000000.0, 22500000000000.0, 25000000000000.0, 28000000000000.0, 32000000000000.0, 36000000000000.0, 40000000000000.0, 45000000000000.0, 50000000000000.0, 56000000000000.0, 63000000000000.0, 71000000000000.0, 80000000000000.0, 90000000000000.0, 100000000000000.0, 112000000000000.0, 125000000000000.0, 140000000000000.0, 160000000000000.0, 180000000000000.0, 200000000000000.0, 225000000000000.0, 250000000000000.0, 280000000000000.0, 320000000000000.0, 360000000000000.0, 400000000000000.0, 450000000000000.0, 500000000000000.0, 560000000000000.0, 630000000000000.0, 710000000000000.0, 800000000000000.0, 900000000000000.0, 1000000000000000.0, 1120000000000000.0, 1250000000000000.0, 1400000000000000.0, 1600000000000000.0, 1800000000000000.0, 2000000000000000.0, 2250000000000000.0, 2500000000000000.0, 2800000000000000.0, 3200000000000000.0, 3600000000000000.0, 4000000000000000.0, 4500000000000000.0, 5000000000000000.0, 5600000000000000.0, 6300000000000000.0, 7100000000000000.0, 8000000000000000.0, 9000000000000000.0, 10000000000000000.0,

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

A resolution test chart featuring a central column of 11 patterns. Each pattern consists of a 3x3 grid of horizontal and vertical lines, with a numerical value to its right. The values are: 4.5, 5.0, 5.6, 6.3, 7.1, 8.0, 9.0, 10, 11.2, 12.5, and 14. To the left of this column are two larger patterns with values 1.0 and 1.1. To the right are two more with values 2.5 and 2.2. Below the central column are three more patterns with values 1.8, 1.25, and 1.4. At the bottom right is a pattern with value 1.6. The patterns become increasingly dense and difficult to distinguish as the numerical value increases.

MICROCOPY RESOLUTION TEST CHART
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