The Effects of Mutations Introduced *In Vitro* into *trbA* and *trbB* on Conjugation in *Escherichia coli* K12

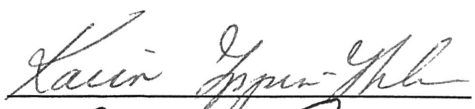
Michael A. Rebolledo

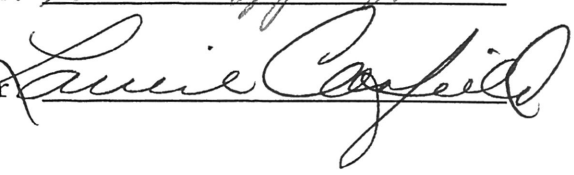
University Undergraduate Fellow, 1986-1987

Texas A&M University

Department of Microbiology

APPROVED

Fellows Advisor: 

Honors Director: 

ABSTRACT

I used an *in vitro* mutagenisis technique to introduce antibiotic gene inserts into two different F plasmid transfer (*tra*) operon genes. The transfer functions of these genes, *trbA* and *trbB*, had not been previously characterized since the genes were discovered only through protein product and DNA sequence analyses of a cloned 2.7 kb F *Pst*I DNA fragment. I started with small clones and insertionally inactivated the *trbA* and *trbB* genes by introducing a gene that encodes antibiotic resistance into a restriction site within their DNA sequence. After confirming the construction of the insert containing plasmids, I transformed each of them into a strain that carries a wild type F*lac* factor and selected recombinant F factors that carried the antibiotic resistance characteristic of the insertion mutation. These recombinants were evaluated for sensitivity to F-pilus specific phages and donor proficiency. My results suggest that insertion of the tetracyline gene into F *trbB* may result in loss of F piliation and transfer capacity, and that the *trbA* gene may not be essential in these functions. However, as non-homologous recombination between F and the pKI plasmids could have occurred to give unexpected configurations of F DNA associated with antibiotic resistance, additional experiments are needed to confirm that the antibiotic resistant F factors I identified do indeed contain *trbA* and *trbB* mutations.

ACKNOWLEDGEMENTS

I would like to thank Dr. Karin Ippen-Ihler for giving me the opportunity to perform this research project. Throughout the past year, her excellent advice kept this project going in the right direction. Her careful revisions during the writing of this paper allowed it to materialize as such. I would also like to thank J. Wu, D. Moore, and P. Kathir for their helpful technical advice. Special thanks go to Jane Lantz for her excellent typing job.

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INTRODUCTION

Evolution has provided bacteria with a broad range of mechanisms to use in order to achieve genetic recombination. Classically, the mechanisms through which new DNA can enter a bacterial cell are known as transformation, transduction, and conjugation. DNA sequences acquired through these processes, together with the occurrence of spontaneous mutations and recombination, have played an essential and fundamental role in the evolution of bacteria in nature (Day, 1982).

This research project was directed towards characterization of a set of genes that specify a conjugation system in the coliform bacteria Escherichia coli K12 (E. coli K12). Conjugation was first described in 1946 by E. L. Tatum and J. Lederberg. They mixed various strains of E. coli K12 together and obtained genetic recombinants. The discovery of this process, known as conjugation, stands out as a landmark in bacteriology and led to an award of the Nobel Prize to Lederberg and Tatum in 1958.

Conjugation involves the unidirectional transfer of deoxyribonucleic acid (DNA) from a donor cell that is in contact with a recipient cell. Although the present study focuses on a particular conjugation system of the Gram-negative organism E. coli K12, a variety of conjugation systems have been described for both Gram-negative and Gram-positive organisms (Clewell, 1981).

The ability of certain E. coli K12 strains to act as donors was soon attributed to the presence of a "sex" or "fertility" factor, later identified as a plasmid known as the F factor. The genes coding for conjugation are carried by this plasmid DNA. Plasmids are extrachromosomal

genetic elements that replicate independently of the host chromosome and are stably inherited from generation to generation. They are ubiquitous in bacterial systems and have been described in some eukaryotic systems as well (Broach et al, 1980). The genetic information that they carry can be responsible for a variety of characteristics; plasmids have been identified that encode antibiotic resistance, colicin production, toxin synthesis or degradation, and metabolic pathways such as nitrogen fixation. Many also specify conjugation systems that cause them to be transmissible from cell to cell. A number of independently isolated plasmids have proven to be F-like in that they encode a conjugation system similar to that of F; many of these, unlike F, confer other easily selectable phenotypes such as antibiotic resistance.

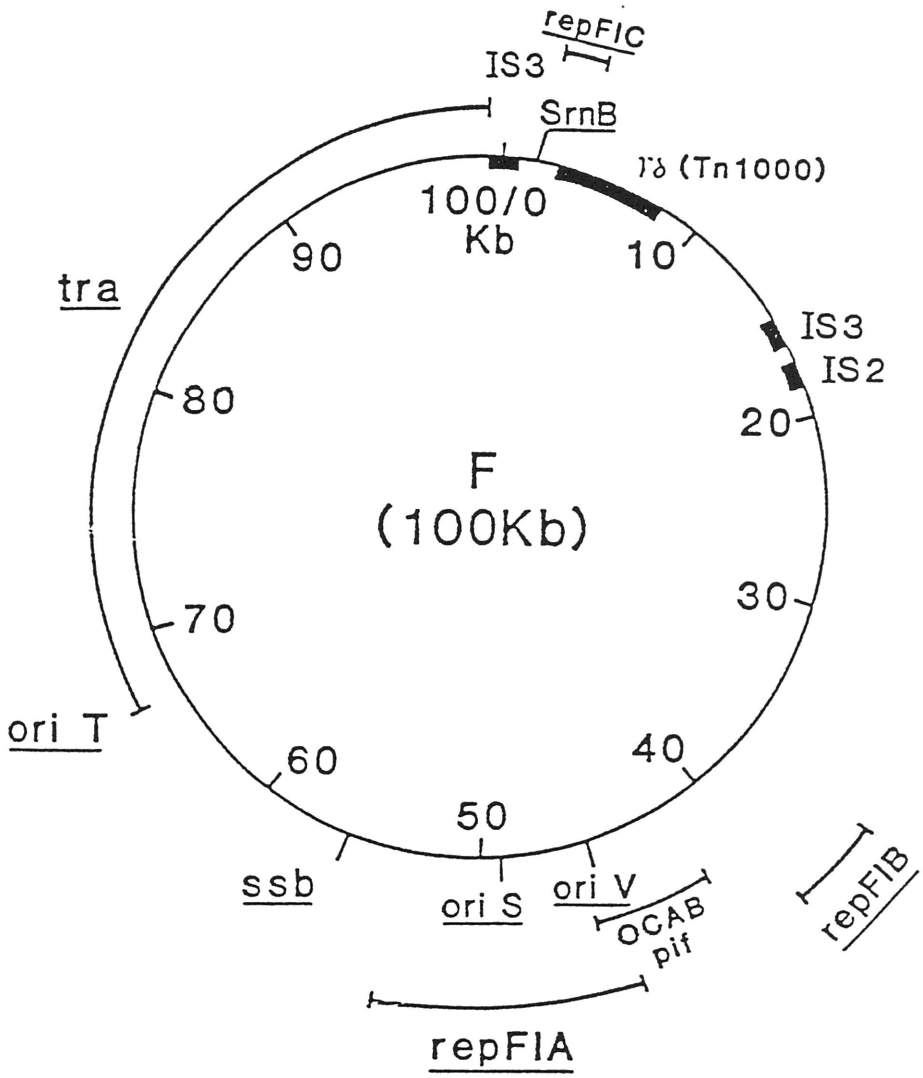
The importance of plasmids in medicine and related fields has become increasingly recognized in the last two decades. Plasmid acquisition is known to be responsible for the spread of multiple antibiotic resistance and drug resistance among pathogenic bacteria. Plasmids can also cause the instability of industrially important microorganisms (Gardner and Snustead, 1984). From an evolutionary standpoint, it is clear that plasmid DNA confers a distinct selective advantage to many organisms.

The F plasmid is a covalently-closed circular, double-stranded piece of DNA nearly 100 kilobases (kb) in length. The F copy number of 1 to 2 plasmid DNAs per chromosome is not directly correlated with chromosomal replication, but is dependent on bacterial growth rate (Glass, 1983). The F genes that encode its conjugation system are located within a 33.3 kb segment of F DNA known as the transfer (<u>tra</u>) region. Most of the genes in this region are transcribed from a single <u>tra</u> operon promoter. The site

where DNA strand displacement and transfer begins during conjugation lies at one end of the transfer region and is known as the origin of transfer or oriT. Other elements on the F plasmid include four Insertion Sequences (an IS1, two IS2 elements and Tn1000). These IS elements have all been well characterized and sequenced (Davidson, et al 1974, Deonier et al, 1983). Their presence determines sites of F::chromosome integration during Hfr formation. Additional genes, involved in F DNA replication or properties such as the inhibition of growth of F$^-$specific bacteriophages, have also been located on F. Willetts and Skurray (1987) have recently devised the 100 kb map of F shown in Figure 1. In total, over 60 gene loci have been identified within the F plasmid.

Over 25 tra operon gene products are required for F transfer to occur. The majority of these essential proteins are involved in the formation of F-pili, long-filamentous appendages that extend an average of 1-2μm from the cell surface and appear to have a central axial hole 2nm in diameter. F-pili, often called sex pili, are morphologically distinct from adhesive fimbriae (common pili) and serve as attachment sites for certain types of DNA and RNA bacteriophages. Only an average of 0.5-1.5 sex pili are present per cell, providing yet another distinguishing characteristic. Sex pili play an essential role in conjugation, but that role is not entirely understood. However, it is thought that sex pili initiate conjugation by extending out from the donor cell surface, interacting with an adjacent recipient cell and, subsequently, retracting back into the donor cell envelope to bring the two cells together. Certain tra gene products that are not required for F-pilus formation are known to be then required to stabilize the interaction between the two cells, while others cause the

Figure 1. The F plasmid
Various genetic loci within F are shown in this diagram.
From Willetts and Skurray 1986.


repFIC
IS3
SrnB
γδ (Tn1000)
100/0
Kb
10
IS3
IS2
20
30
40
50
60
70
80
90
tra
ori T
ssb
ori S
ori V
OCAB
pif
repFIB
repFIA
F
(100Kb)

displacement and transfer of a single strand of DNA to the recipient cell. It is not known whether the hollow sex pili provide the passageway for DNA transfer or whether a pore formed by other tra proteins is involved at this stage. Since a large number of proteins are required to assemble pilin subunits, it is possible that these proteins are in a transmembrane complex that provides the DNA transport pore.

Analysis of mutant phenotypes has permitted the functions of many transfer gene products to be associated with specific stages or events in conjugation. Figure 2 (Ippen-Ihler and Minkley, 1986) groups these into four functions associated with the F tra region: F-pilus formation, stable pair formation, surface exclusion, and DNA transfer. As this map indicates, however, numerous other genetic loci, for which functions cannot be assigned, have recently been identified within the F transfer region. These genes were identified by product or DNA sequence analysis. As no mutations that affect these loci have been available, their requirement in, or contribution to conjugation is unknown. In this work, I have used an in vitro method for introducing mutations into such genes. With this procedure I inserted an antibiotic resistance gene, coding for either kanamycin and tetracycline resistance, into specific sites in two different tra operon genes that were present in a cloned tra operon DNA segment carried on a plasmid vector. After obtaining the in vitro insertions, I constructed strains in which recombination between these plasmid inserts and an F plasmid could occur. I then selected for and identified F factor recombinants that appeared to have acquired the inserted antibiotic resistance gene. I then tested these F plasmids to determine whether the

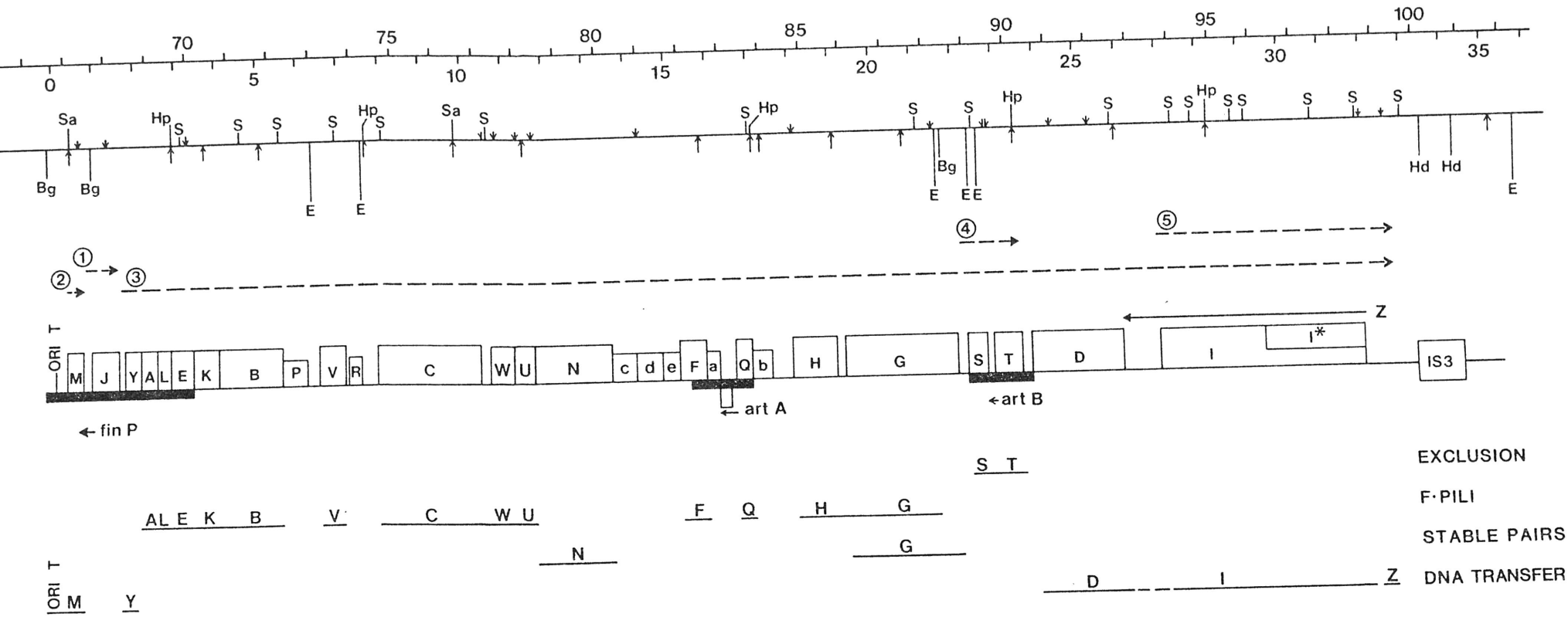
Figure 2. The F transfer region
This figure shows the genetic organization of the 33.3 kb tra operon, (from Ippen-Ihler and Minkley, 1986).

top: Kilobase coordinates for the tra operon on F (65-100; Willetts and Skurray, 1986) or for the tra region (0-33.3) as indicated in the text.

center: The location and size of tra genes is indicated by boxes. Genes of unknown function are shown with short boxes.

bottom: Genes are matched according to their function in conjugation.

Note: The genes trbA and trbB are located in between traF and traH (center of diagram).


70
75
80
85
90
95
100
0
5
10
15
20
25
30
35
Sa
Hp
S
Bg
E
Hd
①
②
③
④
⑤
Z
ORI T
M J Y A L E K B P V R C W U N c d e F a Q b H G S T D I I* IS3
art A
art B
fin P
S T
AL E K B
V
C W U
F Q
H G
N
G
ORI T
M
Y
D I Z
EXCLUSION
F·PILI
STABLE PAIRS
DNA TRANSFER

mutational insert had affected the transfer efficiency or pilus-specific phage sensitivity of the strains carrying the mutant F plasmid.

MATERIALS AND METHODS

Plasmids

pBR322 (Sutcliffe, 1979) was used as a cloning vector in this investigation. J. Sanders provided the *tra* clone designated pKI235. Plasmid pKI235 contains a 2.65 kb *tra* DNA fragment inserted into the pBR322 *Pst*I site. This fragment has been sequenced (J. Wu, unpublished data) and carries four genes of unknown function. A second plasmid, pKI181, provided by June Wu, contains the identical 2.65 kb *tra* fragment inserted into the *Pst*I site of vector pACYC177 (Chang and Cohen, 1978). Plasmid pUC4-KISS, described by F. Barany (1985), carries a kanamycin gene cartridge bounded by multiple restriction enzyme sites. Purified pUC4-KISS DNA was purchased from Pharmacia, Inc. (Piscataway, N.J.). All plasmids used in constructing the mutant *tra* plasmids *in vitro* are briefly described in Table I.

Bacterial Strains and Bacteriophages

Various bacterial strains of *E. coli* were used during the course of this investigation. A list of the strains and their genotypes is given in Table II.

Male-specific and female-specific bacteriophages were used to test for the presence of F-pili and the F plasmid. These bacteriophage lysates were provided by Dr. Ihler's laboratory. The RNA phages Qβ, f2, and R17 adsorb to the side of F-pili. The single stranded filamentous phages f1 and fd attach to the tips of F-pili. Cells that cannot express pilus filaments are resistant to either of these phage types. The "female specific" phage,

Table I: Vectors and Plasmids Used

Plasmid	Kb	Comment
pACYC177	3.9	carries Kanamycin and Ampicillin Resistance genes
pBR322	4.4	carries Tetracycline and Ampicillin Resistance genes
pKI181	6.6	2.65 kb *tra* insert into the *Pst*I site in pACYC177 *amp*
pKI235	7.1	2.65 kb *tra* insert into the *Pst*I site in pBR322 *amp*
pUC4-KISS	4.0	carries Kanamycin Gene Cartridge and Ampicillin Resistance

Table II. Bacterial Strain List

Strain	Genotype
XK5000	F$^-$ *araD139 lacΔU169 rpsL*^{a} *relA thi recA56* (SE5000)
EM9000	F*lac* /*lacΔX74 his trp rpsE*^{b} *tsx ton*
XK1200	F$^-$*lacΔU124* Δ(*nadA gal attλ bio*) *gyrA*^{c}
EM1200	F*lac*/1200
XK3051	F$^-$*lacΔX74 his trp rpsL*^{a} *tsx mal* (λ) (JC3051)

a. *rpsL* strains are Streptomycin resistant.
b. *rpsE* strains are Spectinomycin resistant.
c. *gyrA* strains are Nalidixic acid resistant.

øII, grows very poorly and with low efficiency on strains that carry F. A øII resistant phenotype confirms that an F factor is present even when transfer or piliation is absent.

Media and Antibiotics

All cultures were grown in Luria broth (LB) [10g tryptone, 5g yeast extract, 10g sodium chloride per liter of glass distilled water]. LB plates were made by adding 10g agar to the previous ingredients. LC media was made by adding 0.5ml 20% glucose solution and .25ml 1M$CaCl_2$ per 100ml LB. TYE plates contained 10g tryptone, 5g yeast extract, 5g sodium chloride, and 10g agar per liter of glass distilled water. TYE was the primary media used for culture and purification of strains. However, LB plates were occasionally used with equal success.

Antibiotics were used in LB and TYE media to insure maintenance of antibiotic resistance plasmids. Final concentrations of these antibiotics were as follows: 50μg/ml ampicillin or kanamycin, 15μg/ml tetracycline, 100μg/ml streptomycin, and 20 μg/ml nalidixic acid. Plates containing two different antibiotics such as tetracycline/nalidixic acid or kanamycin/nalidixic acid were used to facilitate screening of transformants and transconjugants.

Lac MacConkey indicator medium was composed of 40g MacConkey agar base, 10g lactose and 1 liter glass distilled water. For conjugative mating tests, M63 minimal media (Pardee et al, 1959) plates were used. This media was supplemented with 2g lactose, 5 mg vitamin B_1, 40 mg histidine, 40 mg tryptophan, and 100 mg streptomycin per liter.

H TOP agar (8g Bactoagar, 10g Bactotryptone, 8g NaCl, 1 liter glass distilled water) was used to plate transformants on TYE plates. F TOP agar (7.5g agar, 8g NaCl, 1 liter glass distilled water) was used to plate transconjugants during conjugative mating tests.

Cloning Methods

Cloning procedures generally followed procedures described by Maniatis et al (1982). Plasmid DNA was isolated using cesium chloride gradient centrifugation as described by Davis et al (1980). Purified plasmid DNA was digested with specific restriction endonucleases. Most restriction endonucleases were purchased from Bethesda Research Laboratories (BRL), however some were also from International Biotechnology, Inc. (IBI), New England Biolab (NEB), Boehringer-Mannheim (B-M), and Pharmacia, Inc.

Restriction endonucleases were used according to product information. Occasionally a double digest made it necessary to readjust the salt concentration of the reaction mixture. A double digest was employed to remove the tetracycline gene from plasmid pBR322. AvaI, a medium salt restriction endonuclease, was used for the initial cut. A medium to high salt buffer (10X) was used to bring the reaction mixture up to a high salt concentration. EcoRI was used to complete the digestion and thus remove the tetracycline gene completely. As AvaI and EcoRI are both sticky-end cutters, the ends of the tetracycline gene fragment were blunted by S1 nuclease digestion (Pharmacia, Inc., Piscataway, N.J.). After BRL introduced their React^{TM} buffer system for restriction endonucleases, restriction digests performed in the latter part of this study took advantage of these buffers.

Agarose gel electrophoresis was used to separate DNA fragments obtained by restriction enzyme digestion. A 1.0% agarose solution was made using 1g Agarose Ultra-Pure DNA grade, 10 ml 10X E buffer (20mM sodium acetate, 40mM Tris base, 50μg ethidium bromide, pH8.0, and 100 ml glass distilled water), and 90ml glass distilled water. Ethidium bromide was added after boiling the solution in a microwave oven. 20ml of the 1% agarose solution was poured on the gel slab and allowed to set. A BRL horizontal gel electrophoresis apparatus (Model H6) was used to carry out the electrophoresis. DNA digests were mixed with sample buffer (0.05% bromophenol, 15% glycerol) in an eppendorf tube prior to gel loading. Usually 10-12μl sample of DNA was used and the volume diluted to 20μl with sample buffer. A 60 volt potential was applied and maintained until the dye front had moved two-thirds down the gel. A polaroid photograph of the gel was taken using a UV light source.

A λcI857 PstI digest was used as a standard to provide migration distances for known DNA fragment sizes. Rf values (the distance the DNA fragment travelled divided by the total distance of the dye front) were calculated for standards and unknowns. A standard curve was drawn on semi-log paper using the standard Rf values. Determination of unknown kilobase fragment sizes was determined by plotting Rf values of unknowns against the standard curve and extrapolating kilobase sizes from the curve.

Prior to ligation, digested DNA was purified by performing a phenol, phenol-chloroform, and chloroform extraction followed by ethanol precipitation. Ligation involved mixing the purified insert and vector in a ratio of 4-5:1 in ligation buffer (50mM Tris pH7.6, 10mM $MgCl_2$, 10 mM DTT, 1mM ATP). A typical ligation mixture would contain 15μl insert, 3μl vector,

4.5μl DNA ligation buffer (5X) and 1 Unit T4 DNA ligase. The ligation reaction was incubated for two hours at room temperature for sticky-end ligations and overnight at 16^{o}C in a shaking water bath for blunt-end ligations. Following incubation, a 1% agarose gel was performed on the ligation mixture to determine if the ligation was successful.

On average, 10-12μl of undiluted ligation mixture was used to transform competent cells. Competent cells were made and transformed according to the methods described by Maniatis et al (1982). Transformants were plated on selective media using H TOP agar (100μl-500μl transformation mixture per 3ml H TOP agar). Strains carrying a plasmid with the desired insert were selected with the appropriate antibiotics.

Those colonies which contained the insert were selected against the original vector phenotype. For example, plasmid pKI149 expressed tetracycline resistance and kanamycin resistance. Colonies expressing the correct phenotype were grown overnight in LB with the appropriate antibiotics. The following day, plasmid DNA was isolated using a rapid plasmid DNA preparation procedure (Holmes and Quigley, 1981). Isolated DNA was purified by phenol extraction and ethanol precipitated. RNA contamination was eliminated by adding 0.2μl of RNAase to each sample. Restriction digests of plasmid DNA were examined to determine the orientation of the antibiotic inserts.

Conjugation Procedure

Tetracycline and kanamycin resistant EM9000 transformants were used to mate with the nalidixic acid resistant strain XK1200. This procedure involved picking a single colony from a selective plate and growing separate standing overnight cultures of both donor and recipient in LB with

antibiotics. In the morning, a portion of the cells was spun down at 7,000 RPM for 10 minutes in a model SS34 centrifuge. Cell pellets were resuspended in LB without antibiotics to give an OD550nm of 0.3. Tubes were incubated for 1 hour to allow cells to recover. Cells by were mated by mixing together 0.1 ml of both donor and recipient cultures with 0.2 ml LB. This mixture was incubated at 37°C without shaking for one hour, diluted and plated on selective TYE plates.

Qualitative Male/Female Bacteriophage Sensitivity Test

This procedure tested the transconjugant's ability to produce F-pili. Standing overnight cultures of the transconjugants, EM1200, and XK1200 were grown without antibiotics in LB. A lawn (0.2ml overnight culture and 3ml H TOP agar) of the strain to be tested was spread on a TYE plate. A 10μl drop of each bacteriophage lysate was spotted in a different quadrant of the lawn. The plates were incubated at 37°C overnight with the bottom of the petri dish sitting on the incubator rack. The following day, the lawns were examined and plaques and plaque morphology was noted.

Qualitative Mating Test

This procedure tested the transconjugant's ability to transfer the F*lac* plasmid to the recipient strain XK3051. Standing overnight cultures of EM1200, X1200, XK3051, and transconjugants were grown without antibiotics in LB. An M63 minimal media plate, described previously, was used to test the transconjugants for conjugative transfer. A lawn (0.1ml overnight culture and 3ml F TOP agar) of XK3051 was spread for each strain to be tested. Various dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) of each donor were spotted in 10μl amounts in various quadrants on each lawn. As a control, an undiluted sample of 10μl from each donor was spotted on a plate without

a lawn of XK3051. A control of an XK3051 lawn by itself was also done. At the end of the procedure, each overnight donor culture was streaked on a Lac MacConkey indicator plate to check for retention of F*lac*. Test and control plates were incubated overnight and checked the next day for *Lac*+ colonies.

Quantitative Plaque Titer for R17 and M13 Bacteriophages

A quantitative plaque titer for R17 and M13 phage lysates was performed using selected transconjugants as hosts. Overnight cultures of transconjugants and EM1200 were grown in LC with the appropriate antibiotics. Cultures were spun individually at 7,000 RPM in a model SS34 centrifuge. The pellets were resuspended in 5 ml LC. R17 and M13 phage lysates were diluted in physiological saline to 10^{-2}, 10^{-4} and 10^{-6}. A 0.1ml sample of each phage dilution was mixed with 0.1ml of each resuspended culture and allowed to stand for 5 minutes at room temperature. 3ml of H TOP was added to this mixture and immediately poured onto a TYE plate. The next day, plaques were counted and the number of plaque forming units (PFU) per ml of phage lysate on each strain was determined.

Quantitative Mating Test

A quantitative mating test was performed using selected transconjugants as donors with the recipient strain XK3051. Overnight cultures of transconjugants and XK3051 were grown in LB with antibiotics. Each culture was spun down at 7,000 RPM for 5 minutes in a model SS34 centrifuge. Donor and recipient cells were resuspended to an OD550nm of 0.6 by diluting with LB. A viable count was done on each culture by plating 10^{-6} and 10^{-7} dilutions (in saline) on TYE plates. Mating was achieved by mixing together 0.2ml of donor and 0.8ml XK3051 in an 18mm test

tube. This mixture was allowed to stand for 1 hour at 37°C. A 0.1ml sample of 10^{-3} and 10^{-4} saline dilutions of the mating mixture was plated on M63 minimal media. Controls of XK3051, EM1200, and each donor culture were streaked separately on M63 minimal media plates. Mating efficiency was determined as: the total number of transconjugants per ml obtained x 100 divided by the total number of viable donor cells present in the mating mixture.

RESULTS

Construction of Mutant *tra* Plasmids *In Vitro*

Construction of a trbB Mutant Plasmid

Plasmid pKI181, shown in Figure 3, was used as the target plasmid for mutagenesis. It contains a 2.65 *tra* fragment (*traF* - *traH*) (See Figure 2). Included in this fragment are four genes of uncharacterized function each of which contains various restriction sites. The *Hpa*I restriction site, located within the *trbB* gene, is a unique restriction site within all of plasmid pKI181. Hence, it provided an ideal site for inactivation of this gene through insertion of an antibiotic resistant gene.

A fragment of DNA encoding tetracycline resistance was obtained from the plasmid pBR322 (Figure 4). An *Eco*RI and *Ava*I digestion of plasmid pBR322 DNA cuts out the gene as a 1.4 kb fragment. I used S1 nuclease treatment to blunt the sticky ends generated by those enzymes. Figure 5 shows a 1.0% agarose gel of plasmids pBR322 and pKI181 digested with *Eco*RI-*Ava*I and *Hpa*I, respectively. Lane 1 contains the λcI857 DNA standard digested with *Pst*I. Lane 2 shows plasmid pBR322 DNA after the *Eco*RI-*Ava*I digestion. The two bands expected, the tetracycline fragment of 1.43 kb, and the remaining DNA fragment of 2.94 kb are present. Lane 3 shows plasmid pKI181 following digestion with *Hpa*I. Since *Hpa*I is a unique restriction site within plasmid pKI181, only one band is expected. The single band of 6.6 kb is indicative of the total size of plasmid pKI181.

Digested DNA was phenol extracted and ethanol precipitated. Ligation was performed by mixing all the fragments together. Following incubation, a 1.0% agarose gel of the mixture indicated that the reaction was complete.

Figure 3. Plasmid pKI181
Plasmid pKI181 is a pACYC177 derivative containing a 2.7 kb tra DNA insert. The unique restriction sites located within the four tra genes (names in parentheses) are indicated. Kan(R): Kanamycin resistance gene, Amp(S): Ampicillin Sensitive

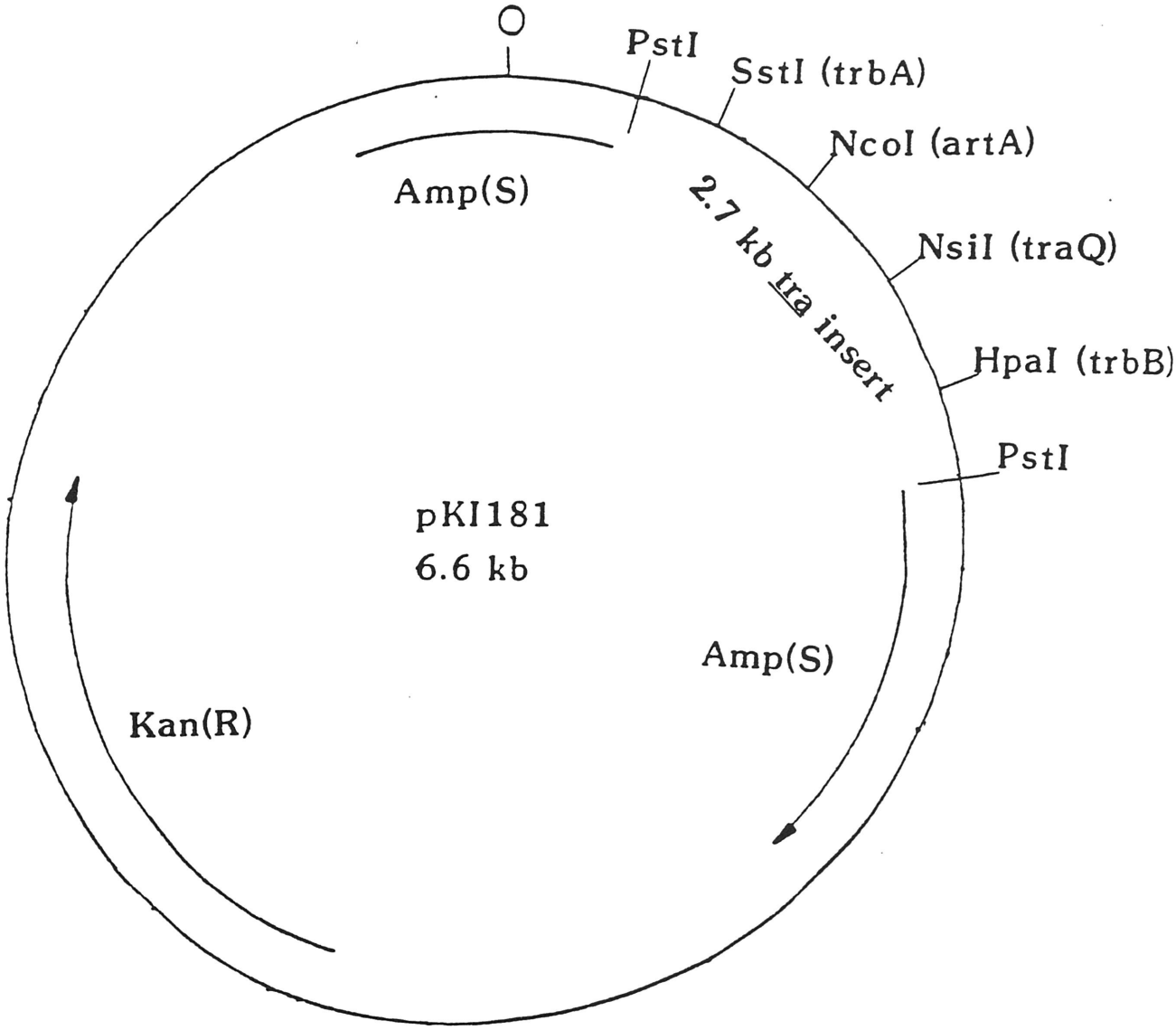

PstI
SstI (trbA)
NcoI (artA)
NsiI (traQ)
HpaI (trbB)
PstI
Amp(S)
2.7 kb tra insert
pKI181
6.6 kb
Amp(S)
Kan(R)

Figure 4. Plasmid pBR322
Plasmid pBR322 is shown with the two restriction sites that were chosen for the removal of the tetracycline gene.
Amp: Ampicillin Resistance Gene
Tet: Tetracycline Resistance Gene

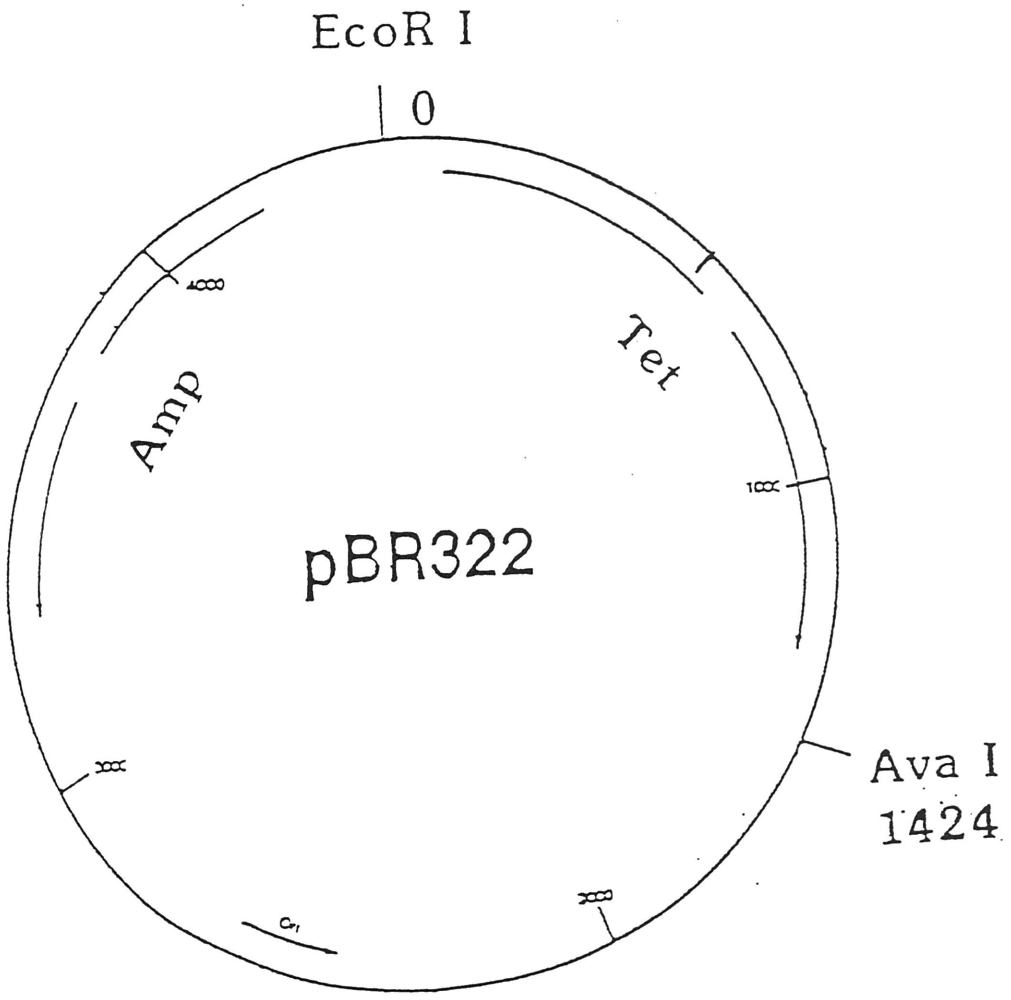
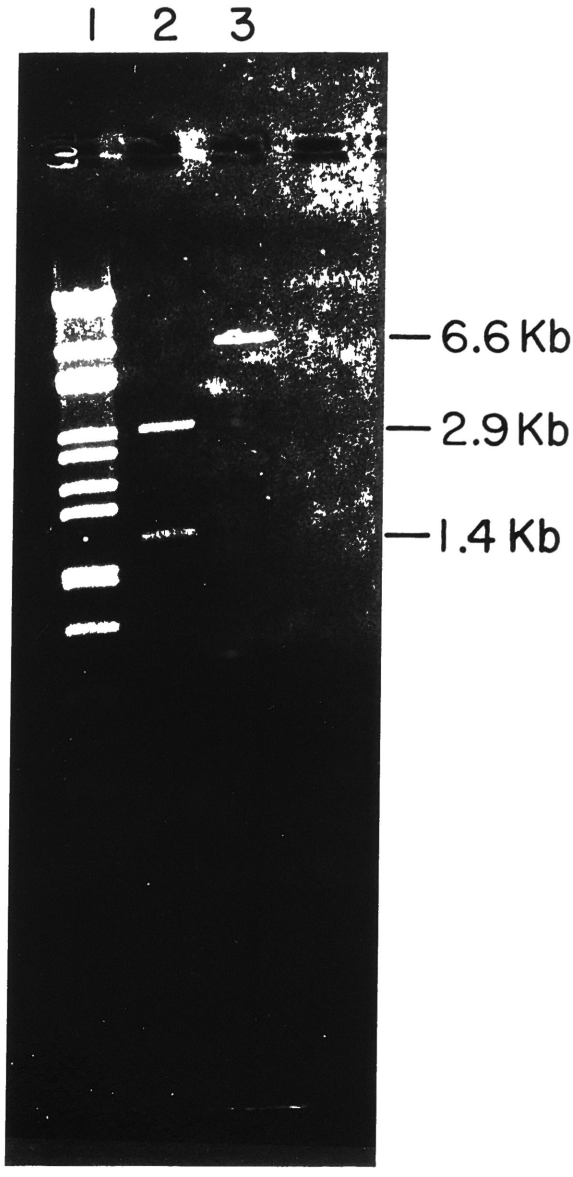

EcoR I
0
Amp
Tet
pBR322
Ava I
1424

Figure 5. A 1% Agarose Gel oF Plasmid pBR322 and pKI181 Restriction Enzyme Digests
This gel photograph shows the various fragment sizes produced by digesting these plasmids with the following restriction endonucleases.
Lane 1: λcI857 PstI fragment standard
Lane 2: pBR322 DNA digested with EcoRI and AvaI
Lane 3: pKI181 DNA digested with HpaI


1
2
3
6.6 Kb
2.9 Kb
1.4 Kb

Competent XK5000 cells were transformed with this ligation mixture and plated on TYE plates containing kanamycin to select for cells that had acquired pKI181 DNA. These kanamycin resistant colonies were then screened for tetracycline resistance to identify plasmids which had acquired the desired insert. Out of 550 screenings, 4 tetracycline/kanamycin resistant colonies were isolated. To confirm that the correct plasmid had been constructed, these four colonies were screened for ampicillin sensitivity. As the tra insert in plasmid pKI181 interrupts the vector ampicillin resistance gene, cells carrying the plasmid pKI181 should be ampicillin sensitive. However, all 4 colonies were found to be ampicillin resistant. This result suggested that such cells contained both pBR322 and pKI181 rather than the desired pKI181 derivative. After screening 50 more tetracycline/kanamycin resistant colonies, an ampicillin sensitive colony was found. This colony was purified several times on appropriate antibiotic plates. The colony, now purified, was saved on an agar slant and in the ultra-freeze (-70°C).

Orientation of the Tetracycline Insert in the trbB Mutant Plasmid

Although the correct phenotype was expressed by these cells, it was necessary to confirm the size and construction of the plasmid DNA present and to determine the orientation of the tetracycline insert. Plasmid DNA was isolated and purified from the strain and then digested with several restriction endonucleases in order to determine the orientation of the insert. The size of this newly constructed plasmid after insertion of the tetracycline gene cartridge is expected to be 8.0 kb. If the insert was positioned in the correct orientation, then upon BamHI digestion two fragment sizes are expected: 2.9 and 5.1 kb. Conversely, if the insert

was found to be in the reverse orientation, then two different fragment sizes are expected: 3.6 and 4.4 kb. Figure 6 shows a 1.0% agarose gel of the plasmid DNA obtained, after digestion with BamHI (Lane 2), EcoRV (Lane 3), and PstI (Lane 4). Lane 1 contains the λcI857 DNA PstI digest used as a standard. Digestion with BamHI produced two fragments: 4.42 and 3.54 kb. These two band sizes indicate that the tetracycline resistance gene is in the reverse orientation to the tra operon fragment. The total size of the new plasmid, as expected, is approximately 8.0 kb. The information obtained from this digestion confirms the size and orientation of the insert in this new plasmid. This plasmid was designated plasmid pKI149 and is diagrammed in Figure 7. Although the tetracycline resistance gene is in the reverse orientation to the direction of transcription through the tra genes, the tetracycline gene has its own promoter which will allow it to be expressed.

Figure 6 also shows two other digestions, not as important as the BamHI digestion, but which are still important to note. Digestion of plasmid pKI149 with EcoRV was performed prior to the knowledge that an EcoRV site expected to be contained in the 2.65 tra fragment was, in fact, not located within this DNA segment. Therefore, as there is only one other EcoRV site in the vector portion of plasmid pKI149, only a single cut was obtained. This gives the single band observed in Lane 2. The fragment can be estimated to be approximately 8.0 kb judging from the results in Lane 1. However, its exact size could not be determined because it lies outside the linear region of the standard curve. The third digestion of plasmid pKI149, that of PstI (Lane 3), produced a thicker than normal band of approximately 4.0 kb. Although this restriction digest reveals no

Figure 6. A 1% Agarose Gel of the Tetracycline/Kanamycin Resistant and Ampicillin Sensitive Plasmid.

This gel photograph shows the various fragment sizes produced after digesting plasmid DNA with BamHI, EcoRV and PstI.
Lane 1: λcI857 PstI fragment standard
Lane 2: plasmid DNA BamHI digest
Lane 3: plasmid DNA EcoRV digest
Lane 4: plasmid DNA PstI digest

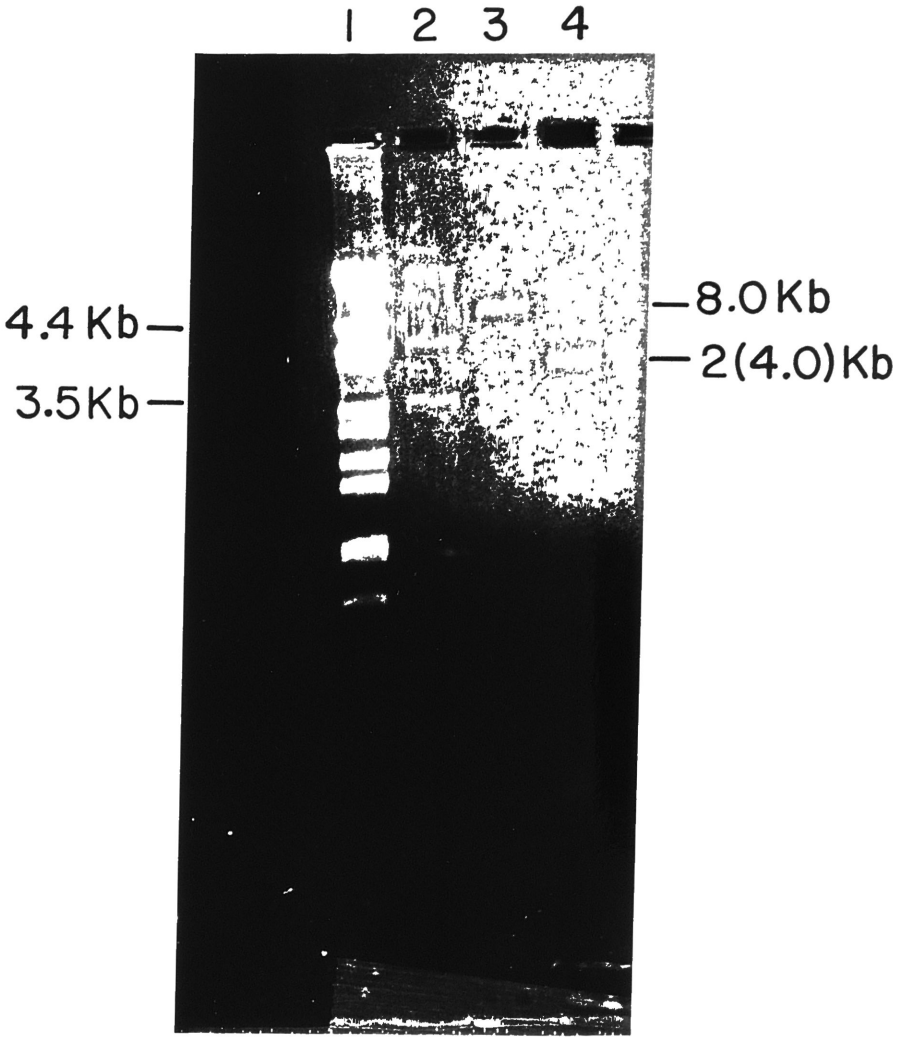
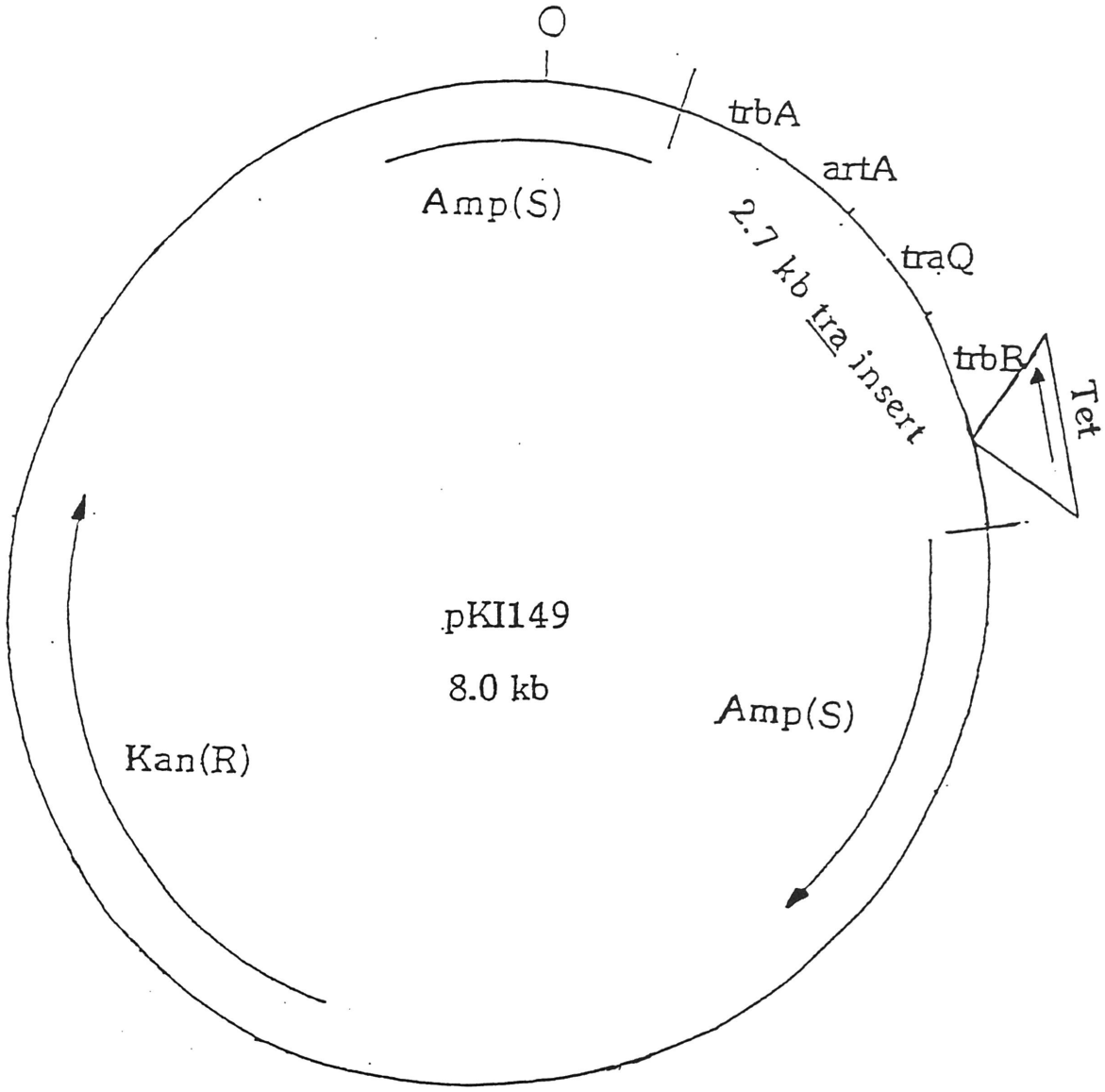

1 2 3 4
4.4 Kb
3.5 Kb
8.0 Kb
2(4.0) Kb

Figure 7. Plasmid pKI149
This figure shows the tetracycline resistance gene insert in the reverse orientation in the trbB gene.
Kan(R): Kanamycin resistance gene
Amp(S): Ampicillin sensitive
Tet: Tetracycline resistance gene


O
trbA
artA
traQ
trbB
Tet
Amp(S)
2.7 kb tra insert
pKI149
8.0 kb
Amp(S)
Kan(R)

information about the orientation of the tetracycline resistance gene, it is consistent with the predicted construction and, size of plasmid pKI149. PstI digestion of pKI181 yields a 2.65 kb tra segment and the 3.93 kb pACYC177 vector fragment. Insertion of the 1.43 kb tetracycline resistance gene into the tra region should increase the size of the 2.65 fragment to 4.08. Thus, as shown in Figure 7, a PstI digest would effectively cut plasmid pKI149 in half, to yield two approximately 4.0 kb fragments that are not distinguishable in size.

Therefore, the analysis of the three restriction digests clearly showed that an 8.0 kb plasmid containing the desired insert had been obtained, and that the transcriptional orientation of the tetracycline insert in pKI149 is opposite that of the tra operon fragment.

Construction of a trbA Mutant Plasmid

For construction of an insertional mutation in trbA I varied the experimental approach slightly to take advantage of the SstI restriction sites available on each side of the kanamycin resistance gene cartridge carried by plasmid pUC4-KISS (see Figure 8). These sites could be used to introduce a "sticky end" SstI fragment expressing kanamycin resistance into the SstI site in trbA. However, since plasmid pKI181 already expresses kanamycin resistance, the trbA insertion could not be selected in this plasmid. Therefore the target plasmid for this experiment was pKI235. This plasmid, like pKI181, contains the 2.65 kb tra DNA PstI fragment. However pKI235 is a pBR322 derivative and expresses tetracycline rather than kanamycin resistance. The structure of plasmid pKI235, is shown in Figure 9. The single SstI restriction site it contains is located within trbA. Therefore, insertion at the SstI site should inactivate the trbA

Figure 8. Plasmid pUC4-KISS
This figure shows the pUC4-KISS plasmid which provided the kanamycin gene cartridge. The gene cartridge is located within the SstI restriction sites.
Amp: Ampicillin resistance gene
Kan(R): Kanamycin resistance gene

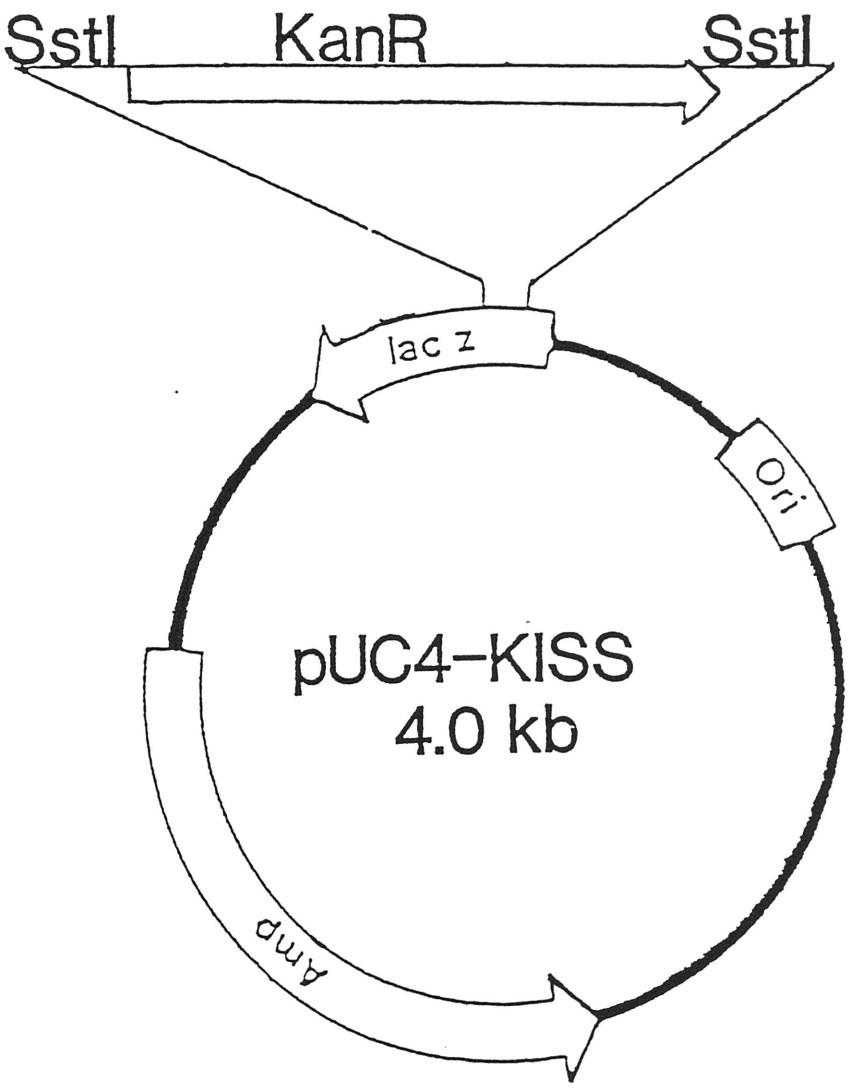
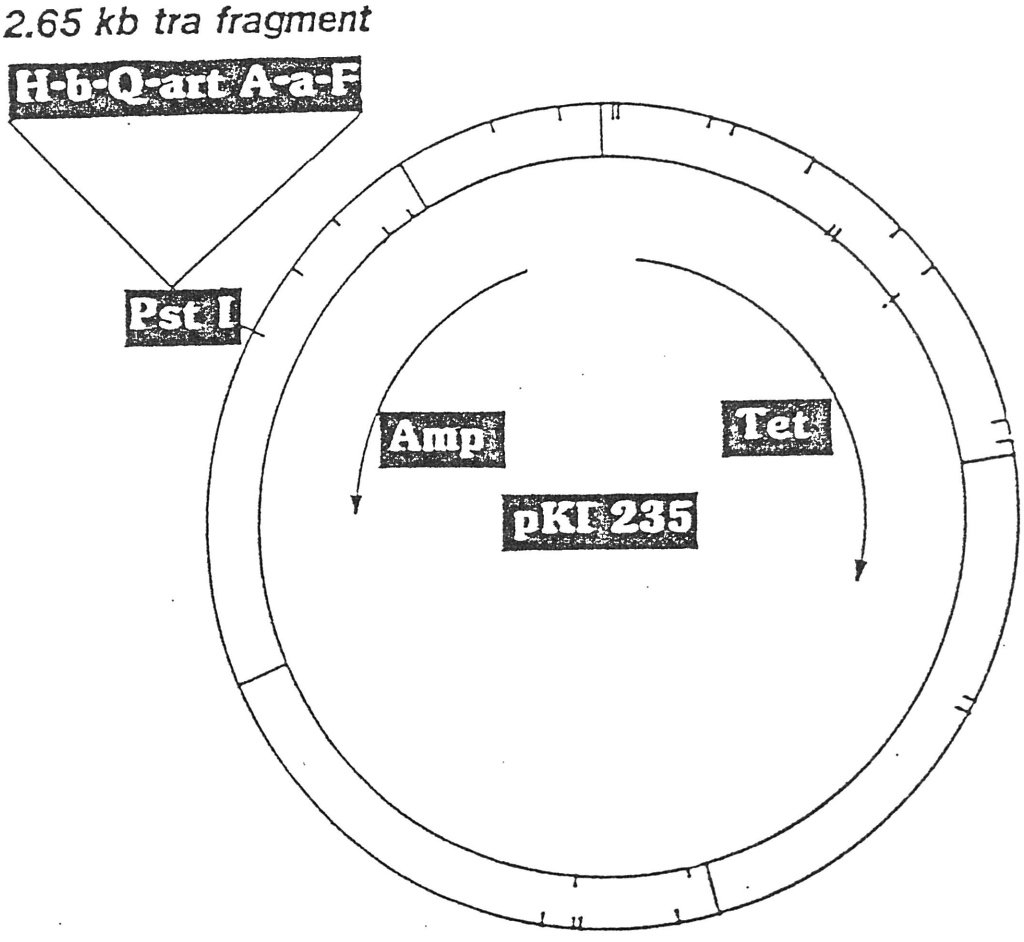

SstI
KanR
SstI
lac z
Ori
pUC4-KISS
4.0 kb
Amp

Figure 9. Plasmid pKI235
Plasmid pKI235 is a pBR322 derivative containing a 2.7 kb *tra* DNA insert. The various *tra* genes are indicated within *Pst*I restriction site. Amp: Ampicillin sensitive, Tet: tetracycline resistance gene


2.65 kb tra fragment
H-b-Q-art A-a-F
Pst I
Amp
Tet
pKT 235

gene.

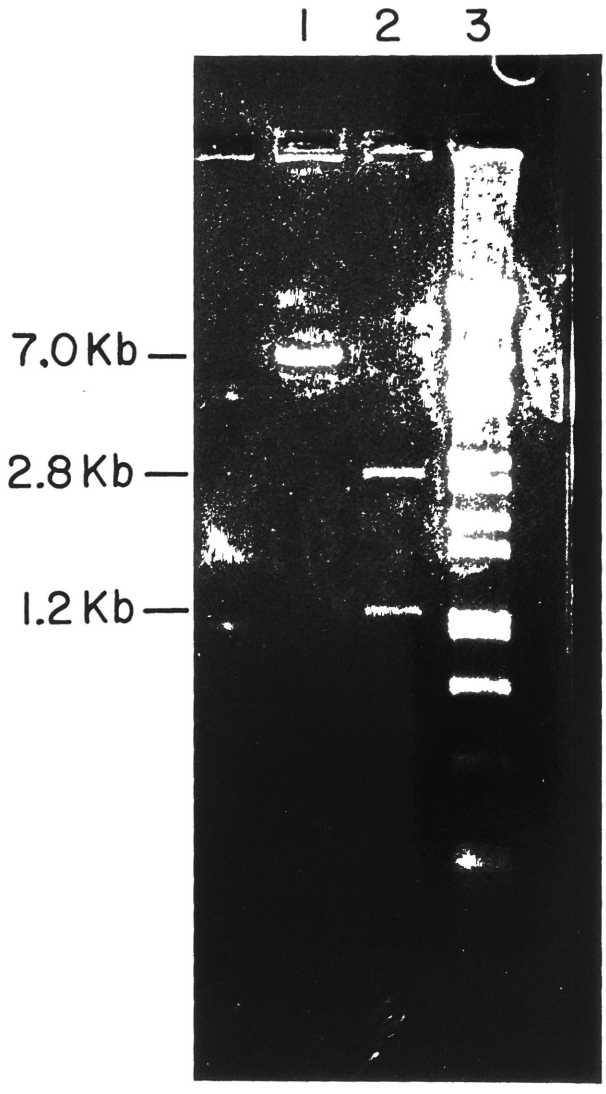
The kanamycin resistance gene cartridge was removed from plasmid pUC4-KISS by an SstI digestion. Likewise, plasmid pKI235 was digested with SstI to open the plasmid. Figure 10 shows a 1.0% agarose gel illustrating the effect of these digestions. Lane 1 shows that SstI digestion of plasmid pKI235 yields the expected single fragment of 7.0 kb equivalent to the total length of pKI235 DNA. Lane 2 shows that the SstI digestion of plasmid pUC4-KISS produced two bands of 2.8 and 1.2 kb. The 1.2 kb fragment represents the kanamycin resistance gene cartridge. Lane 3 contained the standard λcI857 DNA PstI digest.

Ligation was performed by mixing all of the DNA fragments together. Following ligation, an agarose gel was run to confirm that the ligation was complete, and competent XK5000 cells were transformed with the remaining mixture. The desired transformants were selected on TYE plates containing both kanamycin and tetracycline. Approximately 120 kanamycin/tetracycline resistant transformants were screened for ampicillin sensitivity to eliminate cells that carry pUC4-KISS. Six ampicillin sensitive colonies were identified and subsequently purified.

Orientation of the Kanamycin Insert in the trbA Mutant Plasmid

Confirmation of plasmid structure and determination of the orientation of the kanamycin insert was obtained according to procedures similar to those employed for plasmid pKI149. Since there were six clones, however, the probability of finding an insert in each of the two possible orientations increased. Plasmid DNA was purified from each of the six transformants and the restriction endonuclease ClaI was used to determine

Figure 10.A 1% Agarose Gel Showing Digests of Plasmids pKI235 and pUC4-KISS.
This gel photograph shows the fragments produced by digesting these plasmid DNA with the following restriction endonucleases.
Lane 1: plasmid pKI235 SstI digest
Lane 2: plasmid pUC4-KISS SstI digest
Lane 3: λcI857 PstI standard


1
2
3
7.0Kb
2.8Kb
1.2Kb

the orientation of the insert in the kanamycin/tetracycline resistant and ampicillin sensitive plasmids.

Figure 11 shows a 1.0% agarose gel of DNA preparations from the six clones after digestion with ClaI. Also shown in this gel (lane 1) is one sample of an undigested DNA preparation and a λcI857 DNA PstI standard. The purpose of this lane was to insure that the DNA present in other lanes had been cut with ClaI. Lane 2 shows the λcI857 DNA PstI standard. Lanes 3 and 4 show identical band patterns. Lanes 5, 7, and 8 show a different, but identical band pattern. The DNA in Lane 6 appears to have been lost.

The size of this chimeric plasmid containing the kanamycin gene cartridge is expected to be 8.2 kb. If the insert was introduced in the correct orientation, then upon ClaI digestion expected band sizes are 1.6 and 6.6 kb. On the other hand, if the insert was made in the reverse orientation, then two different band sizes are expected: 2.2 and 6.0 kb. The size of DNA fragments in Lanes 3 and 4 were determined to be 5.8 and 2.2 kb, while the bands indicated in Lanes 5,7, and 8 were determined to be 6.4 and 1.6 kb. The band sizes in Lanes 5,7, and 8 were those expected for a plasmid containing the kanamycin gene insert oriented in the same transcriptional direction as the tra operon fragment. One of these plasmid DNAs was designated as plasmid pKI236 (diagrammed in Figure 12). The band sizes in lanes 3 and 4 indicated that these plasmids contained inserts oriented in the reverse direction. One of these plasmids was designated as pKI237 (diagrammed in Figure 13). As in the case of the tetracycline gene fragment used to construct pKI149, the kanamycin gene cartridge inserted into pKI236 and pKI237 contains its own promoter and can be expressed in either orientation. Table III summarizes the specific DNA inserts,

Figure 11. A 1% Agarose Gel Showing ClaI Digests of the Kanamycin/Tetracycline Resistant and Ampicillin Sensitive Plasmids.

Lane 1: uncut plasmid DNA
Lane 2: λcI857 PstI fragment standard
Lane 3-8: ClaI digests of plasmid DNA

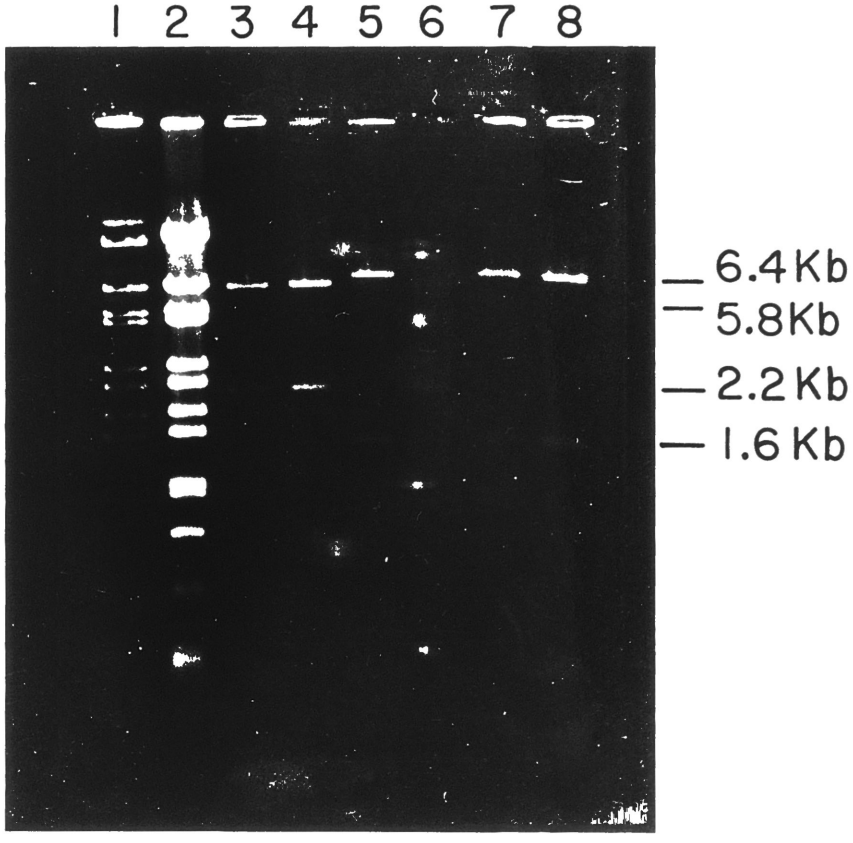

1 2 3 4 5 6 7 8
6.4Kb
5.8Kb
2.2Kb
1.6Kb

Figure 12. Plasmid pKI236
This figure shows plasmid pKI236 with the kanamycin insert in the same orientation as the tra fragment.

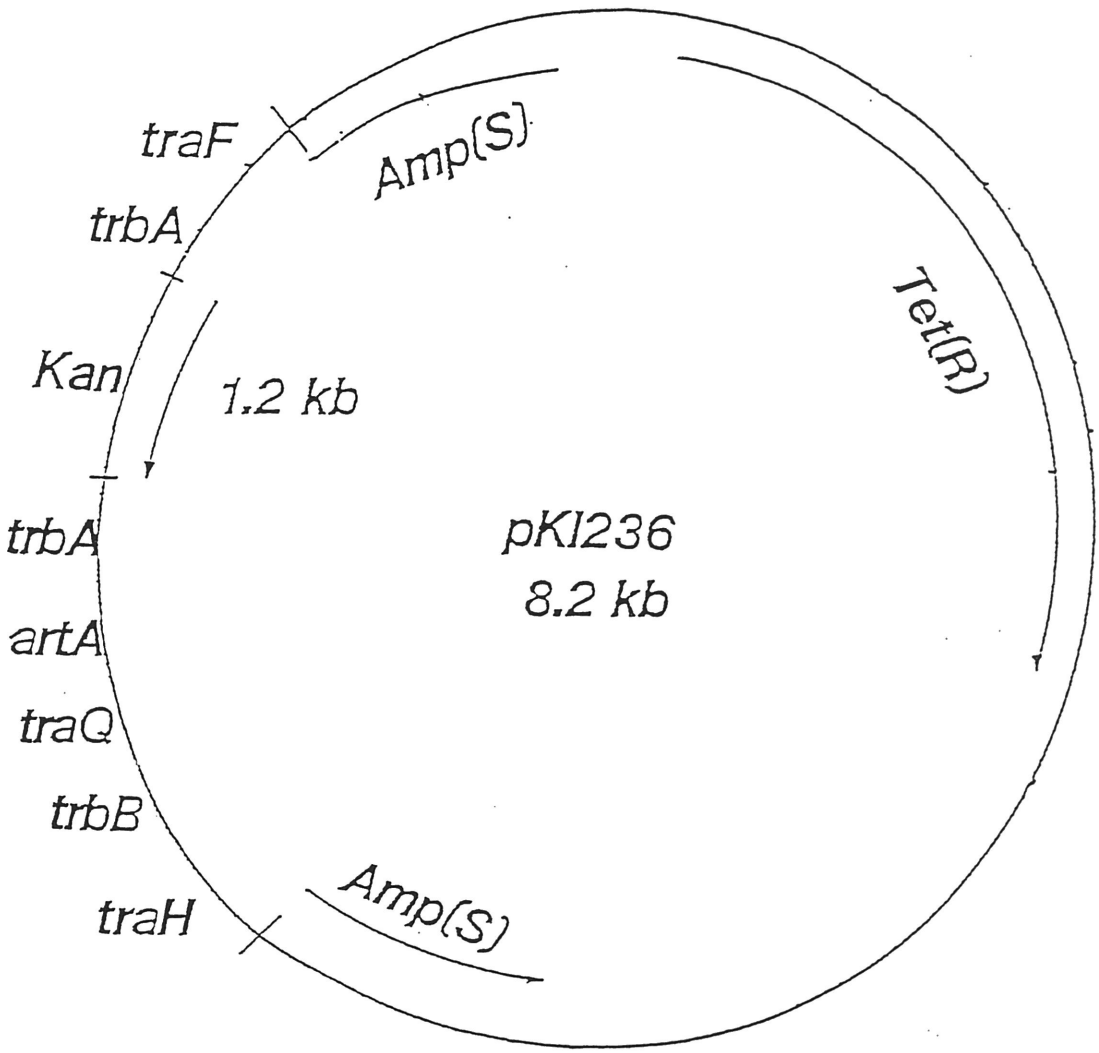

traF
trbA
Kan
trbA
artA
traQ
trbB
traH
Amp(S)
1.2 kb
Tet(R)
pKI236
8.2 kb
Amp(S)

Figure 13. Plasmid pKI237
This figure shows plasmid pKI237 with the kanamycin insert in the opposite orientation of the tra fragment.

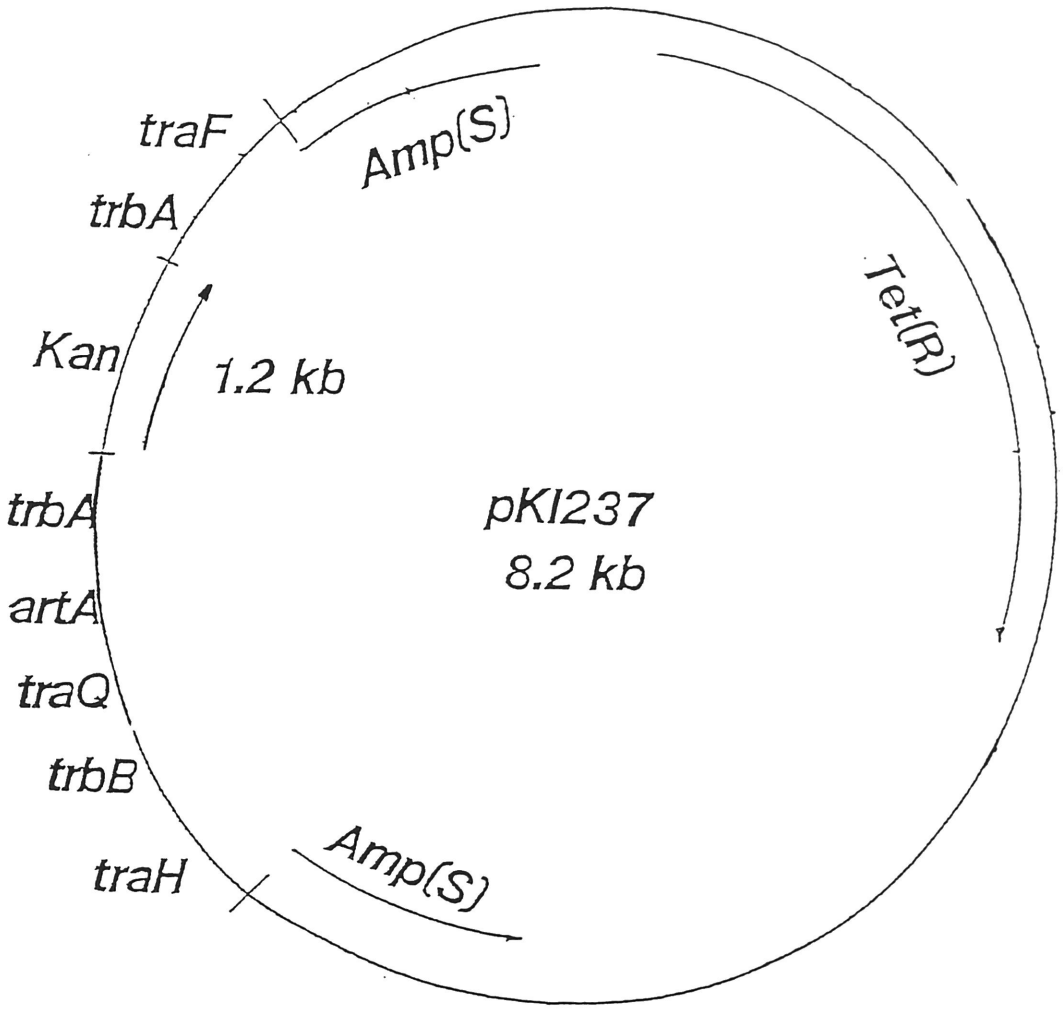

traF
trbA
Kan
trbA
artA
traQ
trbB
traH
Amp(S)
1.2 kb
pKI237
8.2 kb
Tet(R)
Amp(S)

Table III: Construction of Plasmids Isolated

Plasmid Isolated	Insert	Target Plasmid/ Restriction Site		Ligation
pKI149	Tetracycline[a]	pKI181	HpaI	Blunt
pKI236	Kanamycin[b]	pKI235	SstI	Sticky
pKI37	Kanamycin[b]	pKI235	SstI	Sticky

a. From pBR322 EcoRI-AvaI digest treated with S1 nuclease
b. From pUC4-KISS SstI digest

vectors, and restriction sites used to construct plasmids during this study.

Construction of tra Insertion Mutants on the F plasmid

Once antibiotic resistance genes had been inserted into the specific tra operon genes, trbA and trbB carried on vector plasmids, I attempted to recombine these mutations into the intact F plasmid itself. Each mutant plasmid that had been constructed (See Table III) was used to transform a competent culture culture of strain EM9000. This strain carries a wild type Flac plasmid. Recombination should occur between the homologous tra regions on Flac and the chimeric plasmid DNA. A double recombination event occurring on both sides of the antibiotic insert would transfer the insert to the Flac plasmid in EM9000. Although this event is expected to occur at a low frequency, strains carrying the recombinant F factor can be selected by requiring the antibiotic resistant phenotype to be associated with F.

EM9000 transformants carrying pKI149, pKI236 or pKI237 were selected with the appropriate antibiotics. These transformants were mated with XK1200 and transconjugants that had simultaneously received both a lac operon and the antibiotic insert gene were selected. The mating was carried out according to the conjugation procedure described in the Materials and Methods section. Despite the fact that acquisition of the trbA or trbB insertion mutations could lead to loss of Flac transfer ability, we expected that transfer would occur from the transformational donors due to the presence of remaining tra proteins in these cells. Selection of transconjugants eliminated transconjugants that contained only F plasmids that had not undergone recombination. After purification, Lac^+

antibiotic resistant transconjugants were evaluated to determine whether Flac mutant plasmids were present, and to determine whether the phenotype of these plasmids reflected the insertion mutation. An alteration in F transfer properties would thus delineate trbA and trbB functions.

Transconjugants were evaluated initially by two tests: 1. Qualitative Male/Female Bacteriophage Sensitivity Test, 2. Qualitative Mating Test. These two tests gave an indication of whether the transconjugants expressed F-pili, and contained F plasmids capable of transferring. Selected transconjugants that exhibited phenotypic differences from the Flac control strain were also evaluated by a Quantitative Plaque Titer and a Quantitative Mating Test.

XK3051 was the recipient strain used in both Qualitative and Quantitative Mating Tests. EM1200, which contains a normal Flac, was used as the positive (wild-type) control during mating and phage sensitivity tests. XK1200, an otherwise isogenic F^- strain, was used as a negative control.

Transformation of EM9000 and Mating with XK1200

The transformation of EM9000 with the tra mutant plasmid DNAs was very successful and provided hundreds of transformants to use for mating with XK1200. A single colony of each transformant (pKI149/EM9000, pKI236/EM9000, and pKI237/EM9000) was chosen to serve as a donor in the first mating.

A single pKI149/EM9000 transformant colony was used to mate with XK1200. Strain XK1200 contains the chromosomal marker for nalidixic acid resistance. Transconjugants from this mating were selected on plates containing tetracycline and nalidixic acid and screened for kanamycin

sensitivity. Kanamycin resistant transconjugants were eliminated from consideration as this would indicate that pKI149 had been transferred to the recipient along with the F*lac* plasmid through formation of a single F*lac*:: pKI149 cointegrate. Of 100 tetracycline resistant transconjugants screened, four colonies (149-1, 149-2, 149-3 and 149-4) were identified that were tetracycline/nalidixic acid resistant, kanamycin sensitive, and lactose positive. These four colonies thus had the phenotype expected for cells that received an F*lac* *trbB*::*kan* recombinant plasmid.

Similarly, pKI236/EM9000 and pKI237/EM9000 donors were mated with XK1200. However, transconjugants from this mating were selected on kanamycin and nalidixic acid plates and then screened for tetracycline sensitivity on Lac MacConkey plates. Again of 100 transconjugants from the pKI236/EM9000 matings only four colonies (236-1, -2, -3, and -4) exhibited the desired phenotype. In the pKI237/EM9000 mating, 200 kanamycin/nalidixic acid resistant transconjugants were screened, to find 1 colony (237-1) that was also tetracycline sensitive and lactose positive.

Each XK1200 transconjugant that expressed an appropriate phenotype was tested by qualitative mating and bacteriophage sensitivity tests, together with the EM1200 and XK1200 positive and negative control strains. Table IV summarizes the results of these tests.

Analysis of Transconjugants from pKI236/EM9000 and pKI237/EM9000 Donors

The results in Table IV suggested that if the mutation carried by plasmids pKI236 and pKI237 (i.e. the *trbA* insertions) were carried by the F*lac* plasmids in the transconjugants tested, these insertions did not affect the production of F-pili. All four transconjugants from the

Table IV: Qualitative Mating and Bacteriophage Sensitivity Tests for All Transconjugants

Transconjugant	øII[a]	Qβ	R17	fd	f1	f2	Mating[b] (X10^3)
XK1200	Female	R	R	R	R	R	0
EM1200	Male	S	S	S	S	S^W	25
149-1	Male	W	W	W	W	W	35
149-2	Male	S^W	S^W	S^W	S^W	S^W	20
149-3	Male	W	W	W	W	W	20
149-4	Male	S	S	S	S	S	35
236-1	Male	S	S	S	S	S	15
236-2	Male	S	S	S	S	S	4
236-3	Male	S	S	S	S	S	3
236-4	Male	S	S	S	S	S	40
237-1	Male	S	S	S	S	S	6

S: Sensitive; Large plaque indicative of F-pili production
S^W: Weakly Sensitive; Weak plaque with no clear zone
W: Weak; Very Faint light spot
R: Resistant; No plaque at all, indicates absence of F-pili

a. øII phage; A "male" reaction indicates that the F factor is present
b. Number of lactose positive and streptomycin resistant colonies obtained upon mating with XK3051 at the 10^{-3} dilution

pKI236/EM9000 mating would be expected to carry the kanamycin resistance gene in the same orientation as that of F tra operon. These transconjugants still expressed normal sensitivity to both DNA and RNA F-pilus specific phages. The same result was obtained with the single transconjugant from the pKI237/EM9000 mating. This transconjugant would carry the kanamycin gene cartridge inserted into trbA in the opposite orientation to tra operon transcription. Phage sensitivity was equally as strong in this transconjugant.

Although the number of colonies obtained in the transfer test indicated a small variation in transfer frequency among these transconjugants, this mating experiment was not performed quantitatively. Mating in this test was only a qualitative measure of whether or not mating occurred, since a viable count was not performed on either donor or recipient cells.

Lac$^+$ transconjugants that had been acquired by mating the 236(1-4) and 237-1 transconjugants with XK3051 were also checked for kanamycin resistance. These should be kanamycin resistant if the kanamycin gene was stably inserted in F and had been transferred with the F factor again to the XK3051 recipient. Two Lac$^+$ transconjugants from the 10^{-2} dilution of each mating test were screened. All of these Lac$^+$ transconjugants were found to be kanamycin resistant.

Analysis of Transconjugants from pKI149/EM9000 Donors

Table IV indicates that transconjugants 149-1 and 149-3 were less sensitive than normal Flac strains to both DNA and RNA F-pilus specific phages. On the other hand, transconjugants 149-2 and 149-4 exhibited normal phage sensitivities. All four of these transconjugants cultures

appeared able to transfer F*lac* normally. However tests of the Lac^+ colonies obtained showed these were tetracycline sensitive, indicating that the tetracycline gene was not associated with the F factor that was transferred.

Since the 149-1 and 149-3 transconjugants exhibited diminished phage sensitivity, these transconjugants were repurified and evaluated more thoroughly. First, the Qualitative Phage Sensitivity Test on these transconjugants was repeated. These results are shown in Table V.

The results shown strongly suggested that the tetracycline insertion mutation derived from plasmid pKI149 affected sensitivity in both DNA and RNA F-pilus specific phages. Both the 149-1 and 149-3 transconjugants were then evaluated more quantitatively.

Quantitative Mating and Single Plaque Titer for
149-1 and 149-3 Transconjugants

A quantitative mating was performed on transconjugants 149-1 and 149-3. EM1200 was used as the positive control. XK3051 was used as the recipient strain. These results are shown in Table VI.

The results indicate that both the 149-1 and 149-3 transconjugants are reduced in mating efficiency in comparison with the wild type F*lac* control. The mating efficiency of greater than 100% obtained with the latter strain can be attributed to the fact that a four fold excess of females cells was added to the mating mixture, which increases the likelihood of multiple mating events, and that mating was allowed to occur for a 1 hour period at 37°C during which donor and recipient cells could multiply. Lac^+ transconjugants from this mating were found to be 96% tetracycline

resistant, indicating that the purified 149-1 and 149-3 strains contained F factors with stable insertions of the tetracycline resistance gene.

A quantitative plaque titer of R17 and M13 phage lysates was also performed using the same transconjugants. EM1200 was used as the positive control. The R17 titer on EM1200 was 7×10^8 PFU(plaque forming units)/ml, and the M13 titer was greater than 10^9 PFU/ml. No plaques were observed at any dilution on either 149-1 or 149-3. This test clearly indicates a defect in F-pilus production in these mutants.

DISCUSSION

Although a large number of F mutants that are defective in transfer have been isolated, recent characterization of the F transfer operon has shown that none of these affect a number of small genes that are also present within the 33.3 kb transfer region. These genes have been identified only by analysis of the products and DNA sequence of segments of F *tra* operon DNA; their functional contribution to transfer is unknown. It was, therefore, of interest to develop a technique with which mutations could be introduced into these F genes for functional assessment. Since F is a very large, low copy number plasmid and isolation of intact F DNA is difficult, *in vitro* introduction of specific mutations directly into F DNA was not possible. Therefore, the approach taken was to introduce a mutation into a cloned segment of *tra* DNA *in vitro*, and then cross this mutation onto the F plasmid *in vivo*.

In this work, DNA fragments coding for antibiotic resistance genes were introduced *in vitro* into specific sites within a cloned F *tra* region fragment to generate insertion mutations in genes *trbA* and *trbB*. Although many other methods exist for introducing mutations into genes *in vitro*, the method used, in addition to being site specific, has the advantage of resulting in mutations that have a readily selectable phenotype that does not depend upon loss of the function mutated. This was important, because F transfer properties are generally not essential or easily assayable functions, and because the functions of the *trbA* and *trbB* genes were completely unknown. Introduction of an antibiotic resistance gene into these loci on cloned DNAs, thus provided a selectable characteristic that

could be used to derive F*lac* recombinants that carried the same insertion mutation. Furthermore, since the plasmid into which the antibiotic gene was first introduced carried only a small 2.7 kb F *tra* region *Pst*I fragment, unique restriction sites were present in each of the target genes.

The techniques used for introduction of the antibiotic gene into the *tra* DNA clones are standard *in vitro* recombinant DNA technology. Agarose gel electrophoresis analysis of DNA restriction fragments of the plasmids I isolated was used to confirm that the plasmids I constructed contained the desirable mutational inserts as diagrammed in Figures 7, 12, and 13. The most difficult construction was plasmid pKI149. In this case, it was necessary to use S1 nuclease to create blunt ends on the *Eco*RI-*Ava*I pBR322 tetracycline resistance fragment insert before ligation into pKI181; this step was repeated several times before success was achieved. Construction of plasmids pKI236 and pKI237 was easier because, in this case, an *Sst*I fragment was introduced into an *Sst*I site. The ligation reaction was much more efficient because of the homology between the fragment termini. I obtained a larger number of appropriate transformants from the ligation mixture, and was able to identify plasmids containing the kanamycin resistance insert in each of the two possible orientations.

Each plasmid constructed was individually introduced into an F*lac* strain. In the plasmid constructs, the antibiotic gene insert occurs in a known location and is surrounded by the F transfer operon DNA sequences contained in the 2.7 kb *tra* DNA *Pst*I fragment. Therefore, when I transformed these plasmids into the F*lac* strain, EM9000, homologous recombination could occur between the *tra* sequences on F and those

surrounding the antibiotic resistance gene insert. F factor mutants carrying the trbA or trbB insertion mutations will result from this recombination event. Such recombinants can be selected by demanding that the antibiotic resistance encoded by the insert remain associated with the Flac during conjugal transfer. It is important to realize, however, that other recombinational events can occur between the chimeric plasmid and F DNA that result in association of antibiotic resistance with Flac. These arise because F carries one IS1 sequence, two copies of the IS2 sequence and the transposon Tn1000.

In order to obtain an Flac trbB mutant I selected tetracycline and nalidixic acid resistant transconjugants from a mating between the pKI149/EM9000 donor and the nalidixic acid resistant recipient XK1200. That Flac::pKI149 cointegrates were able to form and transfer was apparent from the fact that a majority of these transconjugants were also kanamycin resistant. These obviously contained pKI149 vector sequences and stemmed from illegitimate recombination, and could be eliminated from consideration on the basis of their antibiotic resistance profile. Cultures of the four transconjugants (149-1, 149-2, 149-3, and 149-4) that had the desired antibiotic resistance profile exhibited varied degrees of sensitivity in spot tests with the F-pilus specific phages, and appeared to transfer normally. However, the Lac^+ colonies that I tested from such matings were not tetracycline resistant. This suggested that either the tetracycline resistance gene had been in an unstable association with F that did not survive the second mating, or that the four transconjugant cultures tested contained cells of mixed genotype (wild type and mutant Flac). When colonies from two transconjugants (149-1 and 149-3) that had appeared most

resistant to the pilus specific phages in these initial tests were repurified and tested again more quantitatively. This test made it clear that these strains were fully resistant to infection with the pilus specific phages and very deficient in transfer. This result suggests that the F plasmids in these strains have acquired the trbB tetracyline resistance gene insert and, as a result of this mutation, have lost the ability to express F-pili and to transfer. This would not be an unexpected result, since the trbB locus lies within a cluster of other genes that are involved in F-pilus production (see Figure 2).

However, additional experiments will be required to test this possibility, and to confirm the function of trbB. The most direct test to determine whether the phage resistance and transfer deficiency observed is due to inactivation of the trbB gene will be to transform these strains with pKI181 and observe whether expression of the normal trbB gene supplied by pKI181 will restore phage sensitivity and transfer capacity to 149-1 and 149-3. If so, it will be clear that the only deficiency in the 149-1 and 149-3 Flac donors is in trbB. If the presence of pKI181 does not restore normal donor functions, other possibilities will need to be considered. It would be important to confirm that the tetracycline insertion is indeed in trbB. This could be tested by Southern blot analysis of F factor DNA digests from these strains. It would also be important to test whether expression of tra genes that are distal to trbB has been affected by the trbB insertion. This is a distinct possibility, particularly since transcription from the insert would be occurring in the opposite direction to transfer operon transcription. If the trbB insertion is polar, loss of piliation and transfer could reflect the loss of operon distal gene

products rather than inactivation of trbB. The function of trbB could then only be assessed under conditions where these products are supplied.

In order to obtain an Flac trbA mutation, I selected kanamycin and nalidixic acid resistant transconjugants from EM9000 strains carrying either pKI236 or pKI237. Although the majority of transconjugants again appeared to result from plasmid cointegrates, a total of five kanamycin resistant, tetracycline sensitive transconjugants were identified from these matings. Initial tests of these strains indicated that all were as sensitive to F-pilus specific phages as the normal F control strain, and all appeared to transfer at normal donor frequencies. In all cases, the transfer tests indicated that the kanamycin resistance gene was stably inserted into an F factor that remained transfer proficient, since all Lac^+ transconjugants obtained were also kanamycin resistant. This result seems to suggest that insertional inactivation of the trbA gene with the kanamycin resistance cassette in either orientation does not affect the F functions required for F-pilus formation or transfer. Again, however, additional tests are needed to confirm this result. Since illegitimate recombination events could also give rise to F configurations in which the kanamycin resistance gene is located outside of the F tra operon, the presence of the kanamycin cassette in the Flac trbA gene should be confirmed by Southern blot analysis of F plasmid DNA restriction digests from these strains. It may first also be prudent to test additional transconjugants from the pKI236/EM9000 and pKI237/EM9000 donors to determine whether any transconjugants with altered F phenotypes are discovered.

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