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Characterization of Restriction-Modification Systems in KLEBSIELLA PNEUMONIAE

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LOMA LINDA UNIVERSITY

Graduate School

CHARACTERIZATION OF RESTRICTION-MODIFICATION SYSTEMS
IN *Klebsiella pneumoniae*

by

Boontar Valinluck

A Dissertation in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Microbiology

June 1992

Each person whose signature appears below certifies that this dissertation, in his opinion, is adequate in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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AdoMet	*S*-adenosylmethionine
CFU	Colony-forming unit
Dam	DNA adenine methylation
Dcm	DNA cytosine methylation
EOP	Efficiencies of plating
hsd	Host specificity DNA genes
IPTG	Isopropyl-β-D-thiogalactopyranoside
Mcr	Methylated cytosine restriction
MOI	Multiplicity of infection
Mrr	Methylated adenine recognition and restriction
R-M	Restriction-modification
SDS	Sodium dodecyl sulfate
TE	Tris-HCl-EDTA
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

Bacteria in their natural environment are faced with predation by both macro- and micro-organisms, particularly bacteriophages. They have several extracellular defense mechanisms to prevent attack by bacteriophages, such as the production of a capsule or slime layer, and the mutation of bacteriophage receptors in the cell wall. Once the DNA of the bacteriophages is injected into the bacterial cell, bacteria still have an important intracellular defense mechanism against phages originating from different species of bacteria. This is known as a host-controlled DNA restriction and modification system. The restriction and modification system allows the bacterium the ability to recognize self against non-self DNA. This recognition is possible because the bacterial cell uses its DNA methylation enzymes, which constitute the "modification" part of the system, to methylate specific sites on its own DNA. Any foreign DNA material that does not exhibit methylation at the specific sites characteristic to the host DNA is recognized as non-self and restricted by the host's restriction enzymes, which constitute the "restriction" part of the cell protection system against invading DNA. These phenomena were first observed by Luria and Human (1952) and further characterized by Bertani and Weigle (1953). However, a molecular explanation was first provided by Arber and his colleagues (Arber and Dussoix, 1962; Dussoix and Arber, 1962).

Restriction and modification (R-M) enzymes are common in many bacteria and restriction enzymes have predominately been isolated from bacteria. A few R-M enzymes have been isolated from eukaryotes such as *Sce*I from the yeast

Saccharomyces cerevisiae (Watabe et al., 1983) and *Cre*I from the algae *Chlamydomonas reinhardtii* (Sklar et al., 1986). A recent review (Roberts, 1990) reported the presence of more than 1300 unique restriction enzymes, which suggests the probability that more than 1300 R-M systems of genes may exist. Bacteriophages, on the other hand, have learned to live with the restriction systems of their hosts by developing a wide range of self-defense mechanisms, collectively called "anti-restriction mechanisms", which allow the bacteriophages to avoid the effects of restriction. The different "anti-restriction" mechanisms which bacteriophages have developed include the production of proteins or enzymes to specifically methylate (modify) the bacteriophage DNA; stimulation of the host methylation enzymes to modify the bacteriophage DNA before the host's restriction system is provoked into action; alternatively, the host's restriction endonucleases are inhibited, in addition to the destruction of *S*-adenosylmethionine (AdoMet), which is a restriction endonuclease cofactor (reviewed in Kruger and Bickle, 1983).

The R-M systems are classified into three types (Yuan, 1981) based on the characteristics of the enzymes which function together in the system. The R-M enzymes characteristics include DNA cleavage specificity, structures of the proteins, requirements for catalytic activity (such as ATP, Mg^{2+}, and AdoMet), and DNA recognition sequences of the proteins (Table 1) (Yuan and Hamilton, 1982). The three different R-M systems known today are designated type I, type II, and type III.

Type I and type III are complex multifunctional systems. Modification methylases modify DNA within the recognition site but the restriction

TABLE 1. Characteristics of restriction and modification systems.

Characteristic(s)	Type I	Type II	Type III
Restriction and modification activities	Single multi-functional enzyme	Separate endonuclease and methylase	Single multi-functional enzyme
Subunits for restriction	3	1	2
Subunits for modification	3 or 2	1	1 or 2
Cofactors for restriction	ATP, Mg^{2+}, and AdoMet	Mg^{2+}	ATP, Mg^{2+}, (AdoMet)[*]
Cofactors for modification	AdoMet, (ATP, Mg^{2+})	AdoMet	AdoMet, Mg^{2+}
Other enzyme activities	ATPase, topoisomerase	None	None
Specificity or recognition site	sK:AACN$_6$GTGC sB:TGAN$_8$TGCT	sEcoRI:GAATTC usually two-fold symmetry	sP1:AGACC sHinfIII:CGAAT
DNA methylation site	Specificity site	Specificity site	Specificity site
DNA cleavage site	Random, at least 1,000 bp from specificity site	Specificity site	25-27 bp from specificity site

[*] Compounds in parentheses stimulate activity, but are not required.
(Modified from Yuan and Hamilton, 1982; and Bickle, 1987).

endonucleases cleave outside the recognition sequence (Bickle, 1987). In the type II system, a modification-methylation site and the corresponding restriction site lie within the same specific sequence (Bickle, 1987).

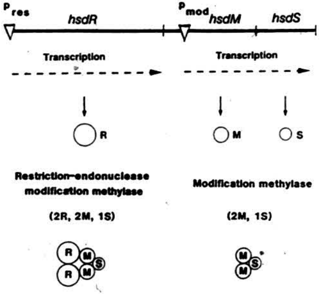
The type I system was the first recognized R-M system. This is the most complex of the three systems and is found in the *Enterobacteriaceae*. So far, a total of eight different type I systems are known in *Escherichia coli* and have been sub-classified into three families known as the "K family" which includes the K, B, and D type I systems of *E. coli* and the "A family" which includes A and E type I systems of *E. coli* (Daniel et al., 1988). The third family consists of plasmid-encoded type I systems, known as EcoR124, EcoR124/3 and EcoDXX1 (Firman et al., 1985; Piekarowicz and Goguen, 1986).

The genes which code for the enzymes of the type I R-M system are referred to as the *hsd* genes (Arber and Linn, 1969). The *hsd* gene cluster is comprised of three structural genes. The *hsdR* gene codes for protein which functions as the restriction enzyme and is responsible for restricting specifically unmethylated (unmodified) DNA. The *hsdM* gene codes for the methylase enzyme which acts to specifically methylate adenosine or cytosine bases, thus modifying the DNA and protecting it from the action of the *hsdR* product. The *hsdS* gene product conveys specificity to the R-M system by allowing the restriction-modification complex to recognize specific sites on the DNA.

The *hsd* genes of type I R-M enzymes have been cloned from several strains of *E. coli* and *Salmonella*. In *E. coli*, the complete *hsd* genes of the K, A, and E systems (Sain and Murray, 1980; Fuller-Pace et al., 1985; Suri and Bickle, 1985), and the *hsdM* and the *hsdS* genes of the B and D systems have been cloned (Gough and Murray, 1983). In *Salmonella*, the *hsdM* and the *hsdS* of the SB, SP, and SQ systems have been cloned (Fuller-Pace et al., 1984). The nucleotide sequence of the entire K system, as well as the nucleotide sequence of the *hsdS* gene of the B, D, SB, SP, and SQ systems has been determined (Gough and Murray, 1983; Gann et al., 1987; Loenen et al., 1987). Interestingly, all type I *hsd* gene clusters have essentially identical gene order, which consists of three genes in the sequence, *hsdR*, *hsdM*, and *hsdS*. There are two promoters in the *hsd* genes (Sain and Murray, 1980); one promoter, P[illegible], directs the transcription of the *hsdR* gene, and a separate promoter, P[illegible], upstream from *hsdM* serves for both the *hsdM* and the *hsdS*; both promoters read in the same direction (Fig. 1).

The type I restriction and modification enzymes in *E. coli* strains K-12 and B have been well studied. The enzymes contain three different subunits, R (MW 135,000), M (MW 62,000), and S (MW 55,000), the products of *hsdR*, *hsdM*, and *hsdS* genes, respectively (Sain and Murray, 1980) (Fig. 1). The type I *hsd* genes are located at 98.5 min on the *E. coli* chromosome map (Bachmann, 1987). Two forms of the enzyme have been recognized (Yuan and Hamilton, 1982). One consists of two M subunits and one S subunit which can perform the modification function only. Another form consists of two R subunits, two M subunits and one S subunit. This form of the enzyme is multifunctional and can function either as a restriction endonuclease or modification methylase depending upon the methylation status of the nucleotides in the recognition sequence. When there is no methylation on either DNA strand at the recognition site, the enzyme will work as an endonuclease; however, it will work as a methylase if only one strand of the DNA is methylated.

FIG. 1. The *hsd*K genes of *E. coli* K-12. The *hsd*K genes, and their direction of transcription are indicated. Three protein subunits, R, M, and S assemble into the two forms of restriction and modification enzymes as shown.


P res
hsdR
P mod
hsdM
hsdS
Transcription
Transcription
R
M
S
Restriction-endonuclease
modification methylase
(2R, 2M, 1S)
Modification methylase
(2M, 1S)
R
R
M
M
S
M
M
S

The *hsdS* gene product is responsible for the binding of the enzyme at a specific recognition site. Several DNA recognition sequences in the type I systems have been identified (Lautenberger et al., 1978; Kan et al., 1979; Sommer and Schaller, 1979; Suri et al., 1984; Nagaraja et al., 1985a, 1985b, 1985c;). All recognition sequences are of a similar pattern consisting of two short, defined regions separated by a spacer of fixed length, but non-specific sequence, as shown in Table 2. Bullas et al. (1976) have reported a unique specificity-site hybrid, SQ, of the *Salmonella* systems SP and SB. Recombination between the *hsdS* sequences of SP and SB gave rise to a hybrid recognition sequence which consists of the upstream 5' recognition domain (3 bp) of SP and the downstream 3' recognition domain (4 bp) of SB (Nagaraja et al., 1985b; Fuller-Pace et al., 1984;). This recombination event occured in the spacer region of the specificity gene. When the recognition domains in SQ were reversed, a new specificity sequence, SJ, was recognized by the invert specificity polypeptide (Gann et al., 1987). The ability to "create" new specificity sites may ultimately allow one to engineer restriction enzymes with designed cutting sites.

There is strong evidence that members of the same family of the type I R-M systems are genetically related to each other (reviewed in Bickle, 1982). DNA hybridization experiments using a probe derived from the *hsd* genes of *E. coli* K-12 have demonstrated a close similarity between the *hsdR* and *hsdM* genes of *E. coli* K-12 and those of several other *hsd* systems in the same family. Their allelic nature, that is the presence of *hsd* genes in the same location on the chromosome has been confirmed. Ryu et al. (1988) reported the possibility of

TABLE 2. Restriction recognition sites of type I and type III R-M systems

Enzyme	Type	Recognition sequence	Reference
*Eco*K	I	AACNNNNNNGTGC TTGNNNNNNCACG	Kan et al., 1979
*Eco*B	I	TGANNNNNNNNTGCT ACTNNNNNNNNACGA	Sommer and Schaller, 1979
*Eco*D	I	TTANNNNNNNGTCY AATNNNNNNNCAGR	Nagaraja et al., 1985c
*Eco*A	I	GAGNNNNNNNGTCA CTCNNNNNNNCAGT	Suri et al., 1984
*Eco*E	I	GAGNNNNNNNATGC CTCNNNNNNNTACG	Cowan et al., 1989
*Sty*SB	I	GAGNNNNNNRTAYG CTCNNNNNNYATRC	Nagaraja et al., 1985b
*Sty*SP	I	AACNNNNNNGTRC TTGNNNNNNCAYG	Nagaraja et al., 1985b
*Sty*SQ	I	AACNNNNNNRTAYG TTGNNNNNNYATRC	Nagaraja et al., 1985a
*Sty*SJ	I	GAGNNNNNNGTRC CTCNNNNNNCAYG	Gann et al., 1987
*Eco*124	I	GAANNNNNNRTCG CTTNNNNNNYAGC	Price et al., 1989
*Eco*124/3	I	GAANNNNNNNRTCG CTTNNNNNNNYAGC	Price et al., 1989
*Eco*DXX1	I	TCANNNNNNNATTC AGTNNNNNNNTAAG	Piekarowicz and Goguen, 1986
*Eco*P1	III	AGACC TCTGG	Bachi et al., 1979
*Eco*P15	III	CAGCAG GTCGTC	Hadi et al., 1979
*Hin*fIII	III	CGAAT GCTTA	Piekarowicz et al., 1981
*Sty*LTI	III	CAGAG GTCTC	De Backer and Colson., 1991

N = any nucleotide, R = either purine, Y = either pyrimidine.
Methylated adenosine residues are underlining boldface.

the existence of a new family of type I R-M system based on complementation studies between eight R-M systems in different *Salmonella* species (all are *serB*-linked *hsd* genes), the K system of *E. coli*, and the SB system of *S. typhimurium*. None of the eight R-M systems complemented the restriction activity of the K system and only two R-M systems, SM (*S. muenchen*) and ST (*S. thompson*), showed weak complementation of the restriction activity of the r^-_{SB} but not the r^-_{K}. In addition, DNA from eight *Salmonella* species was hybridized with *hsd* gene probes from the A and SB systems. Neither probe from the A nor SB systems showed any homology to the DNA from the eight *Salmonella* species. However, a weak homology exists between the various *hsdS* genes in the same family (Nagaraja et al., 1985a; Daniel et al., 1988).

Type II enzymes are the simplest among the three types. The vast majority of the known restriction endonucleases belong to this type. They consist of two distinct proteins, a restriction endonuclease and a modification methylase, both of which specifically recognize common nucleotide sequences which vary widely for different R-M systems. DNA recognition sequences normally contain from 4 to 8 specific nucleotides which may be rotationally symmetric (palindromic) or asymmetric. Type II restriction endonucleases require Mg^{2+} for activity and are routinely used in molecular biology laboratories due to their ability to cut DNA specifically within or near the recognition site. The genes for most of the type II systems have not yet been identified. Some type II genes are located on natural plasmids, such as all the type II restriction endonucleases from *E. coli* (Wilson, 1988a), *Pae*R7I from *Pseudomonase aeruginosa* (Theriault and Roy, 1982), and *Pvu*II from *Proteus vulgaris* (Blumenthal et al., 1985). Other type

II restriction endonucleases are located on the chromosome such as *Dpn*I (*Diplococcus pneumoniae* M), *Dpn*II (*Diplococcus pneumoniae*), *Pst*I (*Providencia stuartii*), *Taq*I (*Thermus aquaticus* YT-1), and *Hha*I (*Haemophilus haemolyticus*) (Wilson, 1988a). However, the possibility that the genes could exist on a large plasmid has not been ruled out. In all systems for which the loci have been mapped, the R-M genes of the same system have been closely linked, but have variable lengths, orders, and orientations (Wilson, 1988a; Wilson and Murray, 1991). Furthermore, no significant homology has been observed in at least 13 type II systems in which the entire R-M genes have been sequenced (Wilson, 1988a).

Type III R-M enzymes consist of four members, P1, P15, HinfIII, and LTI (Arber and Dussoix, 1962; Arber and Waulter-Williems, 1970; Piekarowicz and Kalinoska, 1974; De Backer and Colson, 1991). The restriction endonuclease consists of two different subunits, the products of the restriction and modification genes *res* and *mod*, respectively. This multifunctional enzyme can perform either restriction or modification activity (Hadi et al., 1983; Iida et al., 1983). Whereas, the modification methylase consists of the *mod* gene product. The methylation site is only on the adenine base on one strand of DNA at a specific site which may present a problem for the cell during DNA replication. However, although the mechanism is not yet known in this case, the unmodified DNA is somehow protected. The restriction enzyme requires ATP, as does the type I R-M system. However, ATP is not hydrolyzed in the reaction. The product of the *res* gene of P15 has a MW of 106,000 and is required for restriction only, while the modification gene product of P15 has a MW of 75,000 and is necessary for both restriction and modification (Bickle, 1982; Iida et al., 1983). Iida and his colleagues (1983) also showed that the *mod* gene product provided the sequence specificity for both the restriction and modification reactions, and DNA heteroduplex studies indicated that the restriction genes of P1 and P15 are homologous. The DNA sequences of the P1 and the P15 modification operons were compared and showed that the sequences at the 5' end and the 3' end are highly conserved, but the central sequences are nonhomologous (Humbelin et al., 1988).

A possible type IV R-M system was proposed by Petrusyte and his colleagues (1988) which consists of *Eco*57I in *E. coli* RFL57 and *Gsu*I in *Gluconobacter suboxidans* H-5T. Both restriction endonucleases recognize a hexanucleotide sequence 5'-CTGAAG and 5'-CTGGAG, respectively. The enzymes share similar function with type III enzymes, such as cleaving DNA at a site over 14 nucleotides away from the recognition sequences, Mg^{2+} is required for cleaving, and AdoMet can stimulate their activity. They both differ from type III enzymes in their lack of an ATP requirement, and contain both restriction and modification activities in a single polypeptide (*Eco*57I).

Another less familiar type of restriction, which differs from those three types of R-M systems described above, is a restriction system specific for modified DNA such as that coded for by *mcrA* and *mcrB*. The restriction endonucleases of this type require methylated DNA as substrates for their activity. The bacteria that exhibit this type of restriction do not use methylation as the only mode of identifying "self" DNA. This phenomenon was first described by Luria and Human (1952). They used T-even phages T2 and T6 to test the hypothesis. They observed that the T-even phages propagate poorly in a UDP-glucose-deficient mutant of *E. coli*. In this mutant the DNA is methylated (by the incorporation of 5-hydroxymethylcytosine), however, an additional "modification" of the DNA by glucosylation does not occur (Lehman and Pratt, 1960). Phage DNA propagated in this *E. coli* mutant was restricted, whereupon, if the same phage DNA is propagated in a wild type where both methylation and glucosylation take place, no restriction of the phage DNA is observed. The genes coding for the restriction which may function in this system have been identified at two loci on the *E. coli* K-12 chromosome (Revel, 1967). These genes have been designated *rglA* and *rglB*. These two genes were found later to be identical to the *mcrA* and the *mcrB* genes (modified cytosine restriction) (Raleigh and Wilson, 1986) and their nomenclature has been recently established (Raleigh et al., 1991).

E. coli has been shown to contain at least three restriction systems of this type; McrA protein recognizes and restricts the methylated cytosine in the sequence $5'$-C^{5m}CGG (Raleigh and Wilson, 1986), McrBC proteins recognize and restrict DNA in the methylated sequence $5'$-G^{5m}C (Raleigh et al., 1989), G^{5m}C (Ravel, 1983) or G^{N4m}C (Blumenthal et al., 1985), and Mrr (methylated adenine recognition and restriction) protein recognizes and restricts methylated adenine in the sequence G^{N6m}AC or C^{N6m}AG (Heitman and Model, 1987). The McrC protein is essential for the McrB restriction function (Dila et al., 1990).

Genes for all three systems have been mapped and cloned, and the *mcrB* and *mrr* genes have been sequenced (Raleigh et al., 1989; Ross et al., 1989; Kretz et al., 1991; Waite-Rees et al., 1991). The *mcrB* and *mrr* loci are both located in a 14-kb cluster with the *hsd* genes at about 98.5 min in the following order;

mcrCB-hsdS-hsdM-hsdR-mrr (Raleigh et al., 1989). The *mcrA* gene is located (on the excisable prophage-like element *e14*) near the 25-min locus on the *E. coli* K-12 chromosome and is linked to *purB* (Raleigh et al., 1989).

In contrast, two methylases which are not associated with corresponding restriction endonucleases activity have been identified in *E. coli*. These include the methylase encoded by the *dam* (DNA adenine methylation) gene, which methylates the adenine residue in the GATC sequence (Marinus and Morris, 1973; Geier and Modrich, 1979) and the methylase encoded by the *dcm* (DNA cytosine methylation) gene which methylates the internal cytosine residues in the two sequences CCAGG and CCTGG (Marinus and Morris, 1973; May and Hattman, 1975). Methylation by either Dam or Dcm methylases have been shown to inhibit cleavage of DNA by certain restriction endonucleases whose recognition sequences are either identical to or overlap the recognition sequences of the Dam or Dcm methylase. For example, restriction endonuclease *Mbo*I, whose recognition sequence is GATC, is unable to cleave DNA methylated by the Dam methylase, whereas, *Taq*I restriction enzyme is unable to cut the sequence TCGATC if the adenine is methylated (Backman, 1980; Nelson et al., 1984).

The Dam methylase function is involved in a variety of regulatory mechanisms in *E. coli*, such as gene expression, initiation of chromosome replication and chromosome segregation (Marinus, 1987; Barras and Marinus, 1989). The function of the Dcm methylase is not well understood, however, an involvement in gene regulation, recombination, and repair has been proposed (Marinus, 1984).

Thus, three different categories of genes involving restriction and/or modification have been reviewed: the three classical R-M enzymes (type I, type II, and type III), the restriction systems which require modified DNA as a substrate (*mcrA*, *mcrB*, *mcrC*, and *mrr*), and the modification systems which exhibit no corresponding restriction endonuclease activity (*dam* and *dcm*). The combination of these functions allows cells a variety of modes by which to recognize "self" from "non-self" DNA. These functions are used in self-defense from bacteriophage (Bickle, 1987), as well as protecting the cell's own DNA from its own restriction enzyme (Bickle, 1987) and limiting the free exchange of genetic materials among different species (Arber, 1979).

Whenever a cellular system is being reviewed, cloning is an important tool to further study the genes of interest. Other methods of characterization which include DNA sequencing, identification of a product coded by the cloned gene, mapping, etc., must also be applied. Various approaches have been used to clone the type I *hsd* genes. For example, the *hsd* genes of the K (Sain and Murray, 1980), A (Fuller-Pace et al., 1985), plasmid-encoded EcoDXX1 (Skrzypek and Piekarowicz, 1989) and SB (Fuller-Pace et al., 1984) systems were cloned into λ replacement vectors (referred to here as method I or λ method; Borck et al., 1976). The λ method consists of the following steps. A phage library of recombinants was made first in a non-restricting and non-modifying bacterial host (r^-m^-). The resulting library of phages was plated on the bacterial strain restricting (r^+) for the system sought. The recombinant phages carrying an expressed modification gene modified their own DNA and could propagate in the restricting bacterial host, whereas, other recombinant phages which do not have proper modification would be restricted. The surviving phages were then recovered and further propagated on an r^- host. Subsequently an r^+ host was used and infected with the propagated phages. This cycle was repeated several times (with a final propagation on r^- host) to enrich for clones which carry the modification genes. Finally, the surviving phages were tested for modification activity of the R-M system sought. Once the modification gene was identified, the λ clones were then subcloned into plasmid vectors for further studies. This approach requires two prerequisites. First, a λ-sensitive strain which expresses a restriction (r^+) phenotype of the system sought. If the strain is not available, a new λ-sensitive strain must be constructed. Second, the vector (in this case, λ DNA) must naturally contain a restriction site for the system to be cloned.

The *hsd* genes of the E (Fuller-Pace et al., 1985) system in *E. coli* and the CfrI (Daniel et al., 1988) system in *Citrobacter freundii* were cloned also in λ using a different methodology (method II or DNA hybridization method). As described above, recombinant phage libraries were made first in non-restricting and non-modifying hosts. Subsequently, however, the λ-*hsd* clones were identified by plaque hybridization using the known *hsd* gene of the A system from *E. coli* as a probe. The advantages of this method are that neither a λ-sensitive strain, which express restriction activity, nor a vector containing a cutting site for the system to be cloned are needed. However, several conditions have to be considered to allow the use of this method. First, DNA homology has to be present between the DNA of the bacteria to be cloned and the known *hsd* probes. Thus, a screening for DNA homology between total DNA of the test strain and the known R-M system clones must be done before method II can be applied to the cloning of a gene. Second, since cloning is based solely on the DNA homology, it is possible that only a portion of the *hsd* genes is cloned via the hybridization method and that portion may lack the complete genes required to express restriction or modification activity, thus, further cloning of a larger DNA fragment may be necessary. Third, the clone which contains the entire *hsd* genes may not be able to exist due to self restriction as observed with the LTI system of *S. typhimurium* (described below) (De Backer and Colson, 1991). Finally, a fortuitous DNA homology between the probe and unrelated genes may add an ambiguity to the clone.

In the case of the plasmid-encoded type I R-M systems (EcoR124 and EcoR124/3) and the type III R-M systems encoded by phages P1 and P15 in *E. coli*, the plasmid DNA and the phage DNA were cut with restriction endonucleases (*Hin*dIII or *Bam*HI) and ligated to a plasmid vector (referred to here as method IV or a plasmid method). Transformants were randomly tested for restriction and modification activity of the system sought (Firman et al., 1985; Hadi et al., 1979; Nagaraja et al., 1985; Piekarowicz et al., 1985).

Recently, De Backer and Colson (1991) have cloned the entire region of the *hsd* genes of a new type III restriction-modification member, LTI, of *S. typhimurium*. The cloning was achieved in *E. coli* by using a two-step cloning technique which utilized the procedures of method I and IV. First, the modification methylase clones were selected, based on self-modification activity of the clones. A λ genomic library of $r^-_{LT}m^+_{LT}$ *S. typhimurium* was transfected into a λ sensitive $r^+_{LT}m^+_{LT}$ *S. typhimurium* and the enrichment cycle was repeated several times. Surviving λ clones were tested for modification activity.

Subsequently, the methylase gene in the λ vector was subcloned into pBR328 and transformed into *E. coli*. In the second step, the whole *hsd*$_{LT}$ gene cluster encoding a restriction endonuclease and a modification methylase was cloned into a plasmid, pACYC184, which is compatible with pBR328. A genomic library of the $r^{+}_{LT}m^{+}_{LT}$ strain in pACYC184 was constructed and then transformed into an *E. coli* strain containing the modification methylase obtained from the first step. The clones containing restriction endonuclease activity were selected by their enhanced resistance to non-modified λ phage.

The cloning of type II R-M systems was accomplished by the transfer of restriction and modification genes from the bacteria in which they occur to an *E. coli* host using plasmids as vectors. Two main procedures have been used to isolate *E. coli* clones that carry *hsd* genes from heterogenous populations (reviewed in Wilson, 1988a). The first procedure uses phages to enrich for clones possessing restriction activities. A plasmid library of recombinant clones in *E. coli* was infected with a phage, such as λ, whose DNA contains restriction sites for the system sought. The cells were then plated to recover individual clones that had survived after infection and therefore may carry restriction and, consequently, modification genes. The second procedure was accomplished by using restriction enzymes and enriching for self-modified plasmid DNA *in vitro*. This procedure has been used to clone either individual modification genes or complete R-M systems. Typically, a library was prepared using a plasmid vector that contained the recognition site for the modification methylase, then the plasmid clones were propagated in bacteria which possess no modification activity. In this case, any modification activity detected must arise from the methylase encoded for by genes cloned in the plasmid vector. The plasmids were then purified and the pool of plasmids was digested with a restriction enzyme to which only modified molecules are resistant. Recombinant plasmids expressing modification activity were recovered by transforming the digested plasmid pool back to *E. coli*. So far, more than 100 R-M systems of type II have been completely or partially cloned (Wilson, 1988a; Hammond et al., 1990; Seeber et al., 1990; Dusterhoft et al., 1991; Wilson and Murray, 1991). All the type II R-M systems have been cloned in *E. coli*, except for *Sau*3AI restriction and modification genes of *Staphylococcus aureus*, which were cloned in *Staphylococcus carnosus* TM300 (Seeber et al., 1990). A summary of the cloning methods used to rescue different R-M systems is shown in Table 3.

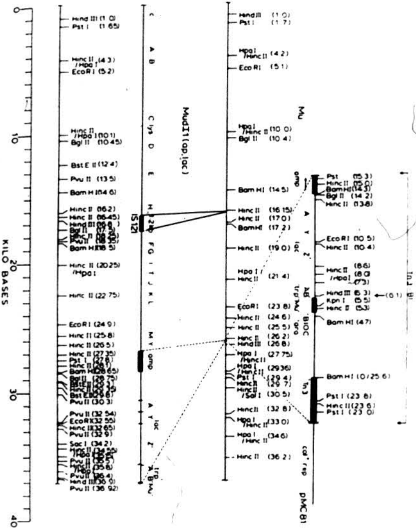
In a different approach to the cloning methodology the transposon-like λ*plac*Mu can be used for cloning genes of interest (method III). λ*plac*Mu, constructed by Bremer et al. (1985), is a derivative of the λ phage which lacks the *attP* (recombination site). λ*plac*Mu also carries the *lacZ*, *lacY*, *Kan*^{r} genes and the insertion sequences from bacteriophage Mu. This phage can be used as a mutator to integrate nonspecifically into the host chromosome, via the Mu transposition mechanism, and create either an operon or protein fusion. Insertion of this specialized transducing phage within a gene of interest can result in the expression of β-galactosidase from the promoter of that gene, however, the gene within which λ*plac*Mu has inserted will be inactivated. The restriction map of the λ*plac*Mu contains a *lacZ* end identical to the *lacZ* end of Mu dl1. The restriction map of the Mu dl1 is now known (Fig. 2). At one terminal next to the *lacZ* gene there is a cutting site for *Eco*RI (O'Connor and Malamy, 1983), thus a fragment

TABLE 3. Examples of the cloning methods used for various R-M systems

Type	R-M system	Cloned gene	Method	Reference
I	K	*hsdMS*	Method I (Lambda)	Sain and Murray, 1980
I	B	*hsdS*	•	Gough and Murray, 1983
I	A	*hsdRMS*	•	Fuller-Pace et al., 1985
I	D	*hsdS*	•	Gough and Murray, 1983
I	E	*hsdRMS*	•	Fuller-Pace et al., 1985
I	SB	*hsdMS*	•	Fuller-Pace et al., 1984
I	SP	*hsdMS*	•	Fuller-Pace et al., 1984
I	SQ	*hsdMS*	•	Fuller-Pace et al., 1984
I	CfrI	*hsdRMS*	•	Daniel et al., 1988
I	EcoR124	*hsdRMS*	Method IV (Plasmid)	Firman et al., 1985
I	EcoR124/3	*hsdRMS*	•	Firman et al., 1985
I	EcoDXX1	*hsdRMS*	Method I (Lambda)	Skrzypek and Piekarowicz, 1989
III	P1	*hsdRM*	Method IV (Plasmid)	Mural et al., 1979
III	P15	*hsdRM*	•	Hadi et al., 1979
III	LTI	*hsdM* *hsdRM*	Method I (Lambda) Method IV (Plasmid)[a]	De Backer and Colson, 1991 De Backer and Colson, 1991
II	Sau3AI	*Sau3AIRM*	Method IV (Plasmid)[b]	Seeber et al., 1990
II	BamHI	*BamHIRM*	Method IV (Plasmid)	Lunnen et al., 1988
II	EcoRI	*EcoRIRM*	•	Greene et al., 1981
II	KpnI	*KpnIRM*	•	Hammond et al., 1990

[a] An *E. coli*, containing an *hsdM*$^{+}$ of LTI in plasmid vector, was used as a recipient.
[b] *Staphylococcus carnosus* TM300 was used as a recipient for cloning.

FIG. 2. Restriction map of Mu dl1 (Ap^r *lac*). Location of genes and restriction endonuclease cutting sites are shown. Number in parenthesis following each restriction enzyme represents the cutting position (in kb) from the C-terminal end of the Mu DNA (From O'Connor and Malamy, 1983).


KILO BASES
0
10
20
30
40
Hind III (1.0)
Pst I (1.65)
Hinc II (4.3)
/Hpa I
EcoR I (5.2)
Hinc II
/Hpa I (10.1)
Bgl II (10.45)
Bst E II (12.4)
Pvu II (13.5)
Bam H I(14.6)
Hinc II (16.2)
Hinc II (16.45)
Hind III (16.8)
Bgl II (17.3)
Hinc II (18.2)
Pvu II (18.35)
Bam H I(18.5)
Hinc II (20.25)
/Hpa I
Hinc II (22.75)
EcoR I (24.9)
Hinc II (25.8)
Hinc II (26.5)
Hinc II (27.35)
Pst I (27.8)
Hinc II (28.1)
Bam H I(28.65)
Bgl II (28.75)
BstE II (29.2)
Hinc II (29.35)
Bst E II(29.8)
Pvu II (30.3)
Pvu II (32.54)
EcoR I(32.55)
Hinc II(32.65)
Pvu II (32.9)
Sac I (34.2)
Hinc II (34.55)
/Hpa I
Pvu II (35.5)
Hinc II (35.8)
/Hpa I
Pvu II (36.4)
Hind III(36.9)
Pvu II (36.92)
C
A B
C lys D
E
H
IS121
2
F G
I T J K L
M Y
amp
A
Y
lac
Z
trp
A B Mu
MudI1(ap,lac)
Mu
Hind III (1.0)
Pst I (1.7)
Hpa I
/Hinc II (4.2)
Eco RI (5.1)
Hpa I
/Hinc II (10.0)
Bgl II (10.4)
Bam HI (14.5)
Hinc II (16.15)
Hinc II (17.0)
BamHI (17.2)
Hinc II (19.0)
Hpa I /
Hinc II (21.4)
EcoRI (23.8)
Hinc II (24.6)
Hinc II (25.5)
Hinc II (26.2)
Hind III (26.8)
Hpa I
/Hinc II (27.75)
Hpa I
/Hinc II (29.36)
Pst I (29.4)
Hinc II (29.7)
Hinc II
/Sal I (30.5)
Hinc II (32.8)
Hpa I
/Hinc II (33.0)
Hpa I
/Hinc II (34.6)
Hinc II (36.2)
amp
A
Y
lac
Z
trp Mu
AB
ori
B10C
Tn3
cal rep
pMC81
Pst (15.3)
Hinc II (15.0)
BamHI (14.3)
Bgl II (14.2)
Hinc II (13.8)
EcoRI (10.5)
Hinc II (10.4)
Hinc II (8.6)
/Hpa I (8.0)
(7.3)
Hind III (6.3)
Kpn I (5.5)
Hinc II (5.3)
Bam HI (4.7)
Bam HI (0/25.6)
Pst I (23.8)
Hinc II(23.6)
Pst I (23.0)
(6.1)
Tn1 81

which contains the *lacZ*-end and portion of the targeted gene can be identified by DNA hybridization with a *lacZ* probe. This structural insertion also allows for further cloning of DNA fragments from the region.

Another method to obtain a clone from the λ*plac*Mu insertion mutant is by UV-induction. UV induces the λ*plac*Mu prophage to excise from its site of insertion. Frequently the λ*plac*Mu excision is "illegitimate" and results in the excision of the neighboring DNA (Bremer et al., 1985).

The problem associated with Mu is its transposition properties which are controlled by two genes, the *A* (transposase) and *B* (involved with the activation of the transposase) genes. Mu transposition includes its duplication and subsequent insert to a new location. Mu is also known to cause DNA rearrangements such as deletions, inversions, duplications, and transposition of host DNA segments (Toussaint and Resibois, 1983). Thus, when a system that utilizes Mu is undertaken, results must be analyzed carefully for deviations from the DNA order and structure found on the chromosome.

A great deal of knowledge related to R-M systems in bacteria has been accumulated by researchers from the department of Microbiology at Loma Linda University. A unique mutant which contains a new hybrid specificity site between SB and SP, named SQ specificity, was isolated (Bullas et al., 1976). Several new restriction and modification systems in *Salmonella* serotypes and also in *K. pneumoniae* strains were found (Pittman, M.S. thesis; Bullas et al., 1980; Bullas et al., 1981). Recombinant strains of *Salmonella* which lost parental recognition specificity have been constructed (Ball, M.S. thesis) and a clone expressing modification activity of the SB system was also isolated (Fuller-Pace et al., 1984). A new family of type I systems has been proposed in *Salmonella* serotypes (Ryu et al., 1988). A mutant (LB5000) and a *galE* derivative (LB5010) of *S. typhimurium* which are r^-m^+ for all three R-M systems (SA, SB, and LTI) were developed (Bullas and Ryu, 1983). Later, a more stable mutant r^-m^+ for all the three systems and *galE*, named JR501, was constructed (Tsai et al., 1989). Furthermore, the effect of restriction on transformation frequency in *S. typhimurium* was evaluated and a quick transformation method for *Salmonella* strains was also developed (Ryu and Hartin, 1990). Recently, operon fusion mutants at the *hsd* gene of K system in *E. coli* K-12 have been constructed (Prakash, M.S. thesis) and the expression and the regulation of the R-M genes were intensively studied (Prakash, M.S. thesis; Reyno, M.S. thesis; Chung, M.S. thesis; Prakash, Ph.D dissertation). However, these studies mainly concentrated on *Salmonella* serotypes and *E. coli*.

Having established an interest in cloning the *hsd* genes, I sought a relatively unexplored R-M system to study. An interest in *Klebsiella* species is rooted in the vast medical importance of this organism. In addition, chromosomal DNA from several *Klebsiella* species has been probed for the *hsd* genes.

Klebsiella species are opportunistic pathogens in a family of *Enterobacteriaceae* that can give rise to bacteremia, pneumonia, urinary tract infection and other types of human infection (reviewed in Montgomerie, 1979). *Klebsiella* species are important nosocomial pathogens (Meers et al., 1981) and can cause life-threatening bacteremia in adults (De la Torre et al., 1985) and in neonates (Morgan et al., 1984). There are seven recognized *Klebsiella* species (Farmer et al., 1985), but only five species (*K. pneumoniae*, *K. oxytoca*, *K. ozaenae*, *K. planticola*, and *K. rhinoscleromatis*) are known to be clinically significant. Of these five, *K. pneumoniae* and *K. oxytoca* are the most important. Characteristically *Klebsiella* species are non-motile, gram-negative rods, surrounded by a thick capsule (Krieg and Holt, 1984). Interestingly, *K. pneumoniae* is only able to fix nitrogen (N_2) in a free living (non-symbiosis) state under microaerobic conditions. This property resulted in the extensive genetic study of nitrogen fixation using *K. pneumoniae* as a model organism. It was found that genes involved in N_2 fixation, *nif* genes, are clustered near the *his* region on the chromosome (Streicher et al., 1971). The genetics and regulation of nitrogen fixation in *Klebsiella* have been reviewed by Magasonik (1982).

Gene transfer in *K. pneumoniae* has been done successfully in the study of the linkage map of the nitrogen fixation (*nif*) genes by bacteriophage P1 transduction (Streicher et al., 1971; Kennedy, 1977) and by conjugation (Dixon and Postgate, 1971). Matsumoto and Tazaki (1971) have also established the genetic linkage map of the *aro* (aromatic acids), *pyr* (pyrimidines) and *pur* (purines) genes in *Klebsiella*. However, genetic studies in *Klebsiella* have been restricted to a few strains.

In the past, transformation of plasmid DNA into *K. pneumoniae* was carried out by the method of Cohen et al. (1972), from which the yield was very low (Espin et al., 1982). A more efficient method which uses a freeze-thaw cycle in the presence of $CaCl_2$ was proposed by Merrick et al. (1987). However, the transformation efficiency of this method depends on the strain used.

Recently, electroporation, a new technology originally used to introduce DNA into eucaryotic cells (Zimmermann and Scheurich, 1981), has been extended to a number of bacterial species (Wirth et al., 1989) and has proven to be very efficient especially in *E. coli* (Dower et al., 1988). In *K. pneumoniae*, the electroporation efficiency reported a transformation efficiency of only 2 x 10^3 CFU/μg DNA, whereas in *K. oxytoca* no transformation was detected, possibly due to restriction by the host strain of the incoming plasmid DNA (Wirth et al., 1989).

Although *Klebsiella* strains were the subject of intensive study for nitrogen fixation, pathogenicity, and epidemiology, the presence of R-M enzymes has not been explored well. One type II R-M system was found in *K. pneumoniae* OK8 (Smith et al., 1976). The restriction endonuclease, *Kpn*I, is now commercially available. Attempts to clone both restriction and modification genes of the KpnI system as a single DNA fragment in a plasmid vector were not successful. All the clones recovered contained a partial deletion at the restriction genes (Hammond et al., 1990). Then a two-step cloning approach was used by first cloning the modification gene in pBR322. The strain with a plasmid carrying the modification methylase was then used as a recipient for transfection with a compatible cosmid library of *K. pneumoniae*. Finally, the clones which contained the entire gene cluster encoding for both the restriction and modification enzymes were obtained and expressed in *E. coli* (Hammond et al., 1990). Several other type II restriction enzymes have been isolated from various strains of *K. pneumoniae*. These include *Kpn*21 (an isoschizomer of *Bsp*MII), *Kpn*K14I (an isoschizomer of *Kpn*I) and various as yet unnamed enzymes which are isoschizomers of *Eco*RII, *Bss*HII, and *Pst*I (Roberts, 1988). Other uncharacterized systems have also been recognized in various strains of *K. pneumoniae* used for the study of nitrogen fixation (Streicher et al., 1974; Satta, personal communication.). Two different R-M systems, originally named KPI and KPII, have been recognized in *K. pneumoniae* M5a1 and *K. pneumoniae* GM236, respectively (Bullas et al., 1981). Based on preliminary studies which will be reviewed in Results, an initial attempt to localize the genes coding for these two systems in the chromosome at the 98.5-min region close to the *serB* marker (all the type I R-M systems are located in this region) has not been successful (Bullas et al., 1981). These results gave rise to the question, where are the genes coding for these two R-M systems located? They may be located at another location on the chromosome or they may be located on a plasmid. In this study these two systems have been renamed as KpnAI and KpnBI with the consultation of R. Roberts (Cold Spring Harbor Laboratory).

What are the characteristics of these R-M systems? Do they belong to type I, type II, type III, or a new type? Do the restriction enzymes of these two systems have any potential commercial use as does *Kpn*I? Would these restriction systems cause problems in transformation as had occurred in *E. coli*? Many questions will arise regarding these relatively uncharacterized systems.

My first goal in this study was to examine if these two systems are unique and different from known R-M systems in *K. pneumoniae*. The second goal was to evaluate the efficiency of several plasmid transformation methods for *K. pneumoniae*, and to examine further the effect of the restriction systems of

K. pneumoniae on plasmid transformation. A restriction system is one of the major obstacles in gene manipulation. A convenient r^-m^+ strain was developed in *E. coli* (Hanahan, 1983) and in *S. typhimurium* (Bullas and Ryu, 1983; Tsai et al., 1989) and widely used. The third goal of this study was to isolate *hsd* mutants from both KpnAI and KpnBI strains and obtain a clue as to how to categorize the two systems. By studying the number of mutants obtained we would expect to see an equal number of r^-m^+ and r^-m^- phenotypic occurrences for any mutagenesis procedure, in the case of the type I and type III R-M systems. However, in the case of the type II R-M system, primarily r^-m^+ phenotypic mutants can be obtained after mutagenesis (Yuan and Hamilton, 1982). My fourth goal in this study was to clone the restriction-modification genes of the KpnBI system which will be the basis of further study of the R-M system.

Further elaboration of this study can lead to the elucidation of both the KpnAI and KpnBI R-M systems in molecular and biological terms. These include sequencing, gene mapping, purification of the gene translational product (enzymes), study of the enzymes, identification of the enzyme recognition sequence, etc. The information obtained will also help to determine the type and family of R-M systems to which KpnAI and KpnBI belong.

MATERIALS AND METHODS

A. Bacterial strains, phages, and plasmids

Bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 4. *Klebsiella pneumoniae* M5a1 and 5022 were obtained from C. Kennedy of the University of Sussex, England, whereas *K. pneumoniae* GM236 and GM238 were kindly provided by G. Satta of the University of Genoa, Italy. The phage used to determine the restriction-modification status of the *K. pneumoniae* strains was isolated from local sewage by L. Bullas of Loma Linda University and designated SBS (Bullas et al., 1981).

B. Media, buffers, and reagents

The formulae for all the media, buffers, and reagents are listed in the Appendix.

C. Restriction and modification tests

Three different methods of testing for restriction and modification activity were used.

(1) *Cross streak method*: This is a qualitative restriction test used for screening a large number of candidates for their restriction phenotype. SBS.0 (propagated on GM238) and SBS.KpnBI (propagated on GM236) at a concentration of 10^7 PFU/ml were streaked on a 1% L agar plate and allowed to dry for a few minutes. Then a single colony of bacteria was suspended in 100 µl B buffer in one well of a 96-well plate and streaked perpendicularly across the

TABLE 4. Bacterial strains, bacteriophages, and plasmids used in this study

Strain	Relevant phenotype and genotype	Source or Reference
BACTERIA		
K. pneumoniae M5a1	$r^{+}_{KpnAI}m^{+}_{KpnAI}$	C. Kennedy; Streicher, 1974
K. pneumoniae M5a1R	$r^{-}_{KpnAI}m^{+}_{KpnAI}$	J. Ryu derived from M5a1 by NTG[*]
K. pneumoniae 5022	$r^{-}_{KpnAI}m^{-}_{KpnAI}$	C. Kennedy; Streicher, 1974
K. pneumoniae GM236	$r^{+}_{KpnBI}m^{+}_{KpnBI}$	G. Satta
K. pneumoniae GM236R	$r^{-}_{KpnBI}m^{+}_{KpnBI}$	J. Ryu derived from GM236 by NTG[*]
K. pneumoniae GM238	$r^{-}_{KpnBI}m^{-}_{KpnBI}$	G. Satta
K. pneumoniae OK8	$r^{+}_{KpnI}m^{+}_{KpnI}$	Roberts, 1988
E. coli NM522	Δ*(lac-proB), hsd*5($r^{-}_{K}m^{-}_{K}$), [F'*proAB*, *lacI*^{q}ZΔM15]	Gough and Murray, 1983
E. coli XL-1 Blue	*hsdR* ($r^{-}_{K}m^{+}_{K}$), [F'*proAB*, *lacI*^{q}ZΔM15, Tn*10* (Tet^{r})]	Stratagene
E. coli CSH50	$r^{+}_{K}m^{+}_{K}$	Miller, 1972
E. coli C	$r^{0}m^{0}$, *dam*$^{+}$, *dcm*$^{+}$	Bertani and Weigle, 1953
E. coli 4001	$r^{+}_{SB}m^{+}_{SB}$	Bullas et al., 1976
E. coli 2379	$r^{+}_{A}m^{+}_{A}$	C. Colson
E. coli 1228 F'JR2	$r^{-}_{K}m^{+}_{K}$	J. Ryu

[*] NTG, nitrosoguanidine

Strain	Relevant phenotype and genotype	Source or Reference
E. coli LE392	$r^-_K m^+_K$, Δ(*lacIZY*)	Stratagene
E. coli P2392	LE392 (P2 lysogen)	Stratagene
E. coli SE5000	Δ(*argF-lac*)U169 *recA56*	Bremer et al., 1984
E. coli MBM7014	Δ(*argF-lac*)U169 *supF*	Bremer et al., 1984
S. typhimurium LT2	$r^+_{SA} m^+_{SA}$, $r^+_{SB} m^+_{SB}$, $r^+_{LT} m^+_{LT}$	Bullas et al., 1976
BACTERIOPHAGES		
λDASH™		Stratagene
λvir		N. Murray
λ*plac*Mu53	*imm* λ'*trp* '*lacZ*⁺*lacY*⁺*lacA*' '*uvrD*'*kan* Mu(cts62) *ner*⁺*A* 'S'	Bremer et al.,1985
λ*plac*Mu507	*cI* ts857 *Sam7* Mu(cts62) *A*⁺*B*⁺	Magazin et al., 1977
M13mp18	*lacZ*'	Bethesda Research Laboratories, Inc.
SBS		Bullas et al., 1981
P1vir		C. Colson
PLASMIDS		
pBluescript™	Ap^r (pUC derivative)	Stratagene
pBR322	Ap^r, Tc^r	New England Biolabs
pGEM-3Z	Ap^r	Promega

Strain	Relevant phenotype and genotype	Source or Reference
pTroy11	*lamB*$^{+}$, Ap^{r}, 6.3 kb in pBR322	De Vries et al., 1984
pRH1	$r^{-}_{K}m^{+}_{K}$, Ap^{r}, 5.8 kb in pBR322	Daniel et al., 1988
pXC1	$r^{-}_{SB}m^{+}_{SB}$, Ap^{r}, 11 kb in pBR322	L. Bullas
pBJ1	Ap^{r}, 7.2 kb in pBluescript	This study
pBJ2	Ap^{r}, 1.2 kb in pBluescript	This study
pBJ3	Ap^{r}, 1.7 kb in pBluescript	This study
pKpnB1	r^{+}_{KpnBI}, Ap^{r}, 6.2 kb in pBR322	This study
pKpnB2	r^{+}_{KpnBI}, Ap^{r}, 5.7 kb in pBR322	This study
pFFP20	Δ*hsd*A, Ap^{r}, 1.1 kb in pBR322	Daniel et al., 1988
pBg3	*hsdR*$^{+}_{K}$ *hsdM*$^{+}_{K}$, Δ*hsdS*$_{K}$	Sain and Murray, 1980
pBg6	Δ*hsdS*$_{K}$	Sain and Murray, 1980
pRH212	$r^{+}_{P1}m^{+}_{P1}$, Ap^{r}, 9.4 kb in pBR322	Mural et al., 1979
pSHI1180	$r^{+}_{P15}m^{+}_{P15}$	Bachi and Arber, 1979
pUNG30	$r^{+}_{EcoR124/3}m^{+}_{EcoR124/3}$, Ap^{r}, 14.2 kb in pBR325	Firman et al., 1985
pRUCL521	Δ*hsdR*$_{LT}$ ($r^{-}_{LT}m^{+}_{LT}$), Ap^{r}, 7.4 kb in pTZ18R	De Backer and Colson, 1991
pRUCL531	$r^{+}_{LT}m^{+}_{LT}$, Cm^{r}, 12 kb in pACYC184	De Backer and Colson, 1991

phages streaks. After drying at room temperature the plates were incubated overnight at 30°C. Bacterial growth only on the streak of phage SBS.0 indicates an r^+ phenotype. On the other hand, the absence of bacterial growth across both streaks of SBS.0 and SBS.KpnBI denotes an r^- phenotype.

(2) *Spot test method*: This procedure is considered to be a semiquantitative method (Colson et al., 1965; Bullas et al., 1980). First, 500 µl of bacteria, grown overnight or at mid-log phase, were mixed with 2.5 ml of soft agar containing both $CaCl_2$ (10 mM) and $MgSO_4$ (10 mM), and then poured onto 1% L agar plates. After letting the soft agar solidify, serial dilutions (10^4, 10^6, 10^8 PFU/ml) of SBS.0 and SBS.KpnBI were dropped onto the bacterial lawn. Once the drops were completely absorbed, the plates were incubated overnight at 30°C unless otherwise stated. The following day, degrees of lysis of SBS.0 and SBS.KpnBI were compared, and the efficiency of plating (EOP) on the test strain relative to the EOP on the non-restricting strain were determined. Furthermore, a modification test could be conducted based on the results of the restriction spot test. To conduct a modification test, a single plaque of SBS.0, formed in a restriction plate, was picked by a needle stab and inoculated into 1 ml of B buffer which was further diluted to 10^{-1} and 10^{-2}. A single drop of the undiluted and diluted phage was spotted onto a lawn of *K. pneumoniae* (r^-) and a lawn of *K. pneumoniae* (r^+) for the tested restriction-modification system. After the drops were absorbed into the agar, the plates were incubated overnight at 30°C.

(3) *Quantitative test*: The test was performed by following the procedure of Bullas et al. (1980). Based on results from the semiquantitative test, 0.1 ml of either SBS.0 or modified SBS phage was added to 0.5 ml of cells cultured

overnight, placed at 30°C for 10 min to allow phage adsorption. A 2.5-ml portion of soft agar containing Ca^{2+} and Mg^{2+} ions was added and poured onto 1% L agar plates. After the soft agar had solidified, plates were incubated overnight at 30°C. The EOP was determined by calculating the ratio of the number of phage plaques on the r^+ strain divided by the number of phage plaques on the r^- strain.

D. Construction of *hsd*KpnBI mutants using λ*plac*Mu

Preparation of λ*plac*Mu *lysate*: High titers of λ*plac*Mu53 and λ*plac*Mu507 (helper phage required for the transposition of λ*plac*Mu53) were prepared by propagating in *E. coli* SE5000 and *E. coli* MBM7014, respectively, and the titer of both λ phages was determined.

Construction of a λ*-sensitive strain of K. pneumoniae GM236 ($r^+_{KpnBI}m^+_{KpnBI}$)*: A plasmid containing the *lamB* gene (this gene codes for the λ receptor protein), pTroy11 (De Vries et al., 1984), of *E. coli* was transformed into *K. pneumoniae* by using a freeze-thaw cycle in the presence of $CaCl_2$ or by electroporation (see section K). Transformants grown on L agar containing ampicillin (1,000 μg/ml) were selected and the presence of pTroy11 was confirmed by plasmid isolation.

Construction of an operon fusion pool: A procedure described by Silhavy et al. (1984) was followed. Ten milliliters of exponential-phase *K. pneumoniae* GM236 carrying pTroy11 were infected with λ*plac*Mu53 (at a multiplicity of infection [MOI] of 1) and λ*plac*Mu507 (at an MOI of 2), and then incubated at 37°C for 30 min without shaking. The bacterial cells were then pelleted and washed three times with 10 ml of fresh L broth. Finally, the cells were suspended in 10 ml L broth containing 10 mM $MgSO_4$, serial dilutions were made and plated on selective media (L agar containing kanamycin 20 µg/ml). After overnight incubation at 30°C, the number of kanamycin-resistant mutants were counted and the transposition frequency was calculated. Meanwhile, the rest of the infected bacterial suspension was incubated overnight for further selection of r^- mutants by conjugation, with *E. coli* 1228 F'JR2 as a donor.

In this study several modified procedures were also tested. The modifications included prewashing bacterial cells; adding both $CaCl_2$ and $MgSO_4$; and the use of different ratios of λ*plac*Mu53 and λ*plac*Mu507 (1:1, 1:2, 1:4, and 1:8).

Conjugation: Donor *E. coli* 1228 F'JR2 was conjugated with the λ*plac*Mu-infected *K. pneumoniae* GM236 (pTroy11) mutant pool as the recipient to enrich for r^-_{KpnAI} mutants. Overnight cultures of both donor and recipient bacteria grown in the appropriate antibiotics were subcultured in 10 ml L broth and grown to an OD_{600} of approximately 0.2 to 0.4. Equal volumes (0.5 ml) of donor and recipient cultures were mixed gently and incubated for 1 h at 37°C without shaking. The sample was then diluted to a required concentration with B buffer and plated on 1.5% L agar plates containing tetracycline (40 µg/ml), ampicillin (1,000 µg/ml), and kanamycin (20 µg/ml). Controls for the donor and recipient bacteria were also plated separately. Following overnight incubation at 30°C, conjugants were tested for the restriction phenotype by cross streaking. The r^- mutants were then confirmed by a drop test.

E. DNA isolation

Isolation of bacterial genomic DNA: Total DNA was isolated from 10 ml of an overnight culture by following the standard technique of Maniatis et al. (1982) with some modifications. The bacterial cells were pelleted at 5,000 rpm (in a Sorvall SS-34 rotor) at 4°C for 10 min and the pellet was suspended in 1 ml of extraction buffer (50 mM Tris-HCl [pH 8.0] and 50 mM EDTA). One milliliter of freshly prepared lysozyme (10 mg/ml in Tris-HCl, pH 8.0) was added and the mixture was incubated in ice water for 45 min. Then 1 ml of extraction buffer, 0.2 ml 10% SDS, and 20 μl proteinase K (10 mg/ml) were added to the sample which was further incubated at 65°C for 1 h. The sample was then extracted twice with phenol:chloroform (1:1) and once with chloroform. The upper aqueous layer was transferred to a new tube, and one tenth volume of RNase A (10 mg/ml) was added and the sample was incubated overnight at 37°C. Again the sample was extracted twice with phenol:chloroform (1:1) and once with chloroform alone. The upper layer was transferred to a 15 ml Corex centrifuge tube. One tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol were added and the mixture was placed at -70°C to precipitate the chromosomal DNA. The DNA was then pelleted at 10,000 rpm (in a Sorvall SS-34 rotor) for 15 min and the pellet was washed twice with 70% ethanol. Finally the pellet was dried in a Savant Speed-Vac Concentrator (Savant Instruments, Inc., Farmingdale, N.Y.), and the DNA was dissolved in 1 ml TE buffer. For evaluation of the DNA concentration, 10 μl of the dissolved DNA was diluted with 990 μl of TE buffer and the absorbance at 260 nm was read. The A_{260} of 50 mg DNA/ml was assumed to be 1.0 (Maniatis et al., 1982).

A small scale genomic DNA extraction, from 1.5 ml of bacterial culture was prepared by proportionally scaling down the above procedure.

Isolation of plasmid DNA: A small-scale and a large-scale protocols were performed in this study. The small-scale method was used to check for the presence of plasmid DNA in transformants after cloning and subcloning. The large-scale method was used for preparation of larger amounts of plasmid DNA that could be utilized for further studies, such as the preparation of radioactively-labeled probes.

Small-scale plasmid preparation: The method of Ausubel et al. (1987) was used with a minor modification. A 1.5-ml portion of an overnight culture was pelleted in a microfuge tube, the bacterial cells were then suspended in 100 µl of lysozyme solution (5 mg/ml lysozyme, 9 mg/ml glucose, 10 mM EDTA, and 10 mM Tris-HCl, pH 8) and kept on ice for 30 min. A 200-µl volume of an alkaline-SDS solution (0.2 M NaOH and 1% SDS) was added and mixed by vortexing briefly. The sample was incubated on ice for 5 min, then 150 µl of 3 M sodium acetate (pH 4.9) was added and the incubation was continued for another 30 min with occasional shaking. The sample was then centrifuged for 10 min at 4°C in a table-top Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.). The supernatant was transferred to a new tube and two volumes of cold absolute ethanol were added to precipitate the DNA. After a 10 min incubation at -70°C for 10 min, the DNA was pelleted by centrifugation in the microcentrifuge at 4°C for 15 min and washed twice with 70% ethanol. The DNA pellet was then dried in the Savant Speed-Vac Concentrator and dissolved in 50 µl of TE buffer.

Large-scale plasmid preparation: A quick, large-scale plasmid preparation protocol of Krieg and Melton (Promega Note, March 1985) was used with a slight modification. An overnight culture of bacteria in 250 ml of selective medium containing applicable antibiotic was pelleted at 5,000 rpm (in a Sorvall SS-34 rotor) at 4°C for 15 min. The pellet was suspended in 6 ml of freshly prepared lysozyme solution (2 mg/ml lysozyme in 25 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 15% sucrose) and incubated on ice for 20 min. Next, 12 ml of alkaline-SDS solution (0.2 M NaOH and 1% SDS) were added, mixed thoroughly by inversion, and the mixture was kept on ice for 10 min. After centrifugation at 15,000 rpm (in a Sorvall SS-34 rotor) for 15 min, the supernatant was transferred to a new tube; 50 μl of RNase A (1 mg/ml) were added and the mixture was incubated for 20 min at 37°C. The mixture was extracted twice with phenol:chloroform (1:1) and once with chloroform only. The upper aqueous layer was transferred to a new tube and two volumes of absolute ethanol were added to precipitate the DNA. After incubation at -70°C for 15 min, the DNA was pelleted by centrifugation, at 10,000 rpm (in a Sorvall SS-34 rotor) for 10 min and washed twice with 70% ethanol. The pellet was dried in the Savant Speed-Vac Concentrator and then dissolved in 1 ml TE buffer.

Isolation of λ phage DNA: A high titer of a λ phage lysate (at least 10^{10} per ml) was prepared by using a "thick-agar" plate method. First, a single plaque is transferred as an agar plug with a sterile Pasteur pipette and crushed in 200 μl of SM buffer (see Appendix). A 500-μl sample of an appropriate bacterial host (OD_{600} = 0.1 to 0.2) grown in L broth containing 10 mM $MgSO_4$ and a 2.5 ml portion of melted top agar were added to the phage suspension. The mixture was then poured on top of a freshly prepared, thick (10 millimeters), L agar plate and incubated at 37°C until complete lysis was observed (approximately 12 to 16 h). The phage lawn was collected by scraping the soft top agar and centrifuged at 10,000 rpm (in a Sorvall SS-34 rotor) for 10 min at 4°C to removed the agar. The supernatant was then transferred to a new tube and treated with 0.3% chloroform for 1 h.

DNA was isolated from the lysate using a Lambdasorb™ kit (Promega, Madison, Wis.). First, 15 µl of Lambdasorb (which is made up of *Staphylococcus aureus* cells coated with rabbit antibodies against λ phage) was mixed with 150 µl of the λ lysate at room temperature for 30 min. The bacterial cells were pelleted by centrifuging in an Eppendorf microcentrifuge at 4°C for 15 min and washed twice with 1 ml of SM buffer (see Appendix). The pellet was then suspended with 0.2 ml of TE buffer and heated at 67°C for 5 min to release the phage DNA. After 5 min of centrifugation to remove the Lambdasorb, the supernatant was collected in a fresh tube containing 2 µl of 5 M NaCl. The sample was then extracted twice with one volume of phenol:chloroform (1:1), and once with chloroform. The aqueous phase was transferred to another tube. An equal volume of 5 M ammonium acetate and two volumes of absolute ethanol were added to the mixture which was placed at -20°C for at least 15 min. The DNA was then pelleted in the microcentrifuge for 30 min at 4°C and washed twice with 70% ethanol. The pellet was dried in the Savant Speed-Vac Concentrator and dissolved in 20 µl of TE buffer.

F. Restriction endonuclease digestion

Type II restriction enzymes and reaction buffers were obtained from either Bethesda Research Laboratories, Inc. (Gaithersberg, Md.) or New England Biolabs, Inc. (Berverly, Mass.) and used as recommended by the manufacturer.

G. Agarose gel electrophoresis

Two different horizontal submerged gel electrophoresis apparatus were routinely used in this study.

A mini-gel electrophoresis apparatus (7 by 10 cm) was used for separating small amounts of DNA and for monitoring the effect of digestions by endonucleases. A mini-gel, made of 0.8% agarose in Tris-acetate-EDTA buffer plus ethidium bromide (10 μg/ml), was submerged in an electrophoresis apparatus containing the same Tris-acetate-EDTA buffer plus ethidium bromide. The DNA samples and DNA markers were loaded in each well and electrophoresis procedure at 30 to 50 volts for 1 to 3 h depending on the size of the DNA fragment and the separation desired.

A larger-scale gel electrophoresis apparatus (14.5 by 20 cm) was used for longer electrophoresis and better resolution of DNA fragments. Gels run using this apparatus were used for Southern blotting. The gel was made of approximately 200 ml of 0.8% agarose in Tris-acetate-EDTA buffer containing ethidium bromide. After loading DNA samples and markers, the gel was usually run for 12 to 18 h at 30 to 50 volts.

At the end of the electrophoreses, a photograph was taken using a Polaroid camera model DS34, high speed coaterless black and white Polaroid 667 film and UV-light illumination (Foto/Phoresis model I [Fotodyne, New Berlin, Wis.] for a mini gel, and model Foto UV30 [Fotodyne] for a large gel). The distance of DNA migration was measured with a fluorescent ruler.

H. Southern blotting

Southern blotting (Southern, 1975) is a technique used to transfer DNA from an agarose gel to a nitrocellulose or nylon membrane (for multiple probing) placed in contact with the gel. The procedure was performed as follows: The DNA in the gel was first denatured with 400 ml of 1x denaturation buffer for 45 min at room temperature and then neutralized with 400 ml of 1x neutralizing buffer for another 45 min. The gel was placed on a filter paper wick which had been set over a glass plate which was the same length as the gel and saturated with 10x SSC. Next, a sheet of pre-wet nitrocellulose membrane (cut to the same size as the gel) was placed directly over the gel and plastic wrap was placed on the upper and lower edge of the gel (about 0.5 cm of overlapping). Four sheets of 20x SSC-saturated filter paper were placed on top of the membrane and followed with a six-inch stack of flattened paper towels. Another glass plate was placed on the paper towels and then a 1-kg weight was placed on top. The blotting was performed overnight. Upon completion of the blotting procedure, the membrane was marked at each well position, carefully removed and baked at 80°C for 2 h.

I. DNA-DNA hybridization

Prehybridization: A 1x prehybridization mixture was made by diluting 2 volume of 2.5x prehybridization solution with 2 volumes of deionized formamide and one volume of distilled water. The mixture was heated for 10 min in boiling water, quickly chilled in ice water and poured into a container. A baked membrane was placed in the mixture, the container was tightly closed and incubated at 42°C with slow shaking at approximately 50 rpm for at least 6 h. This membrane was then used for the following hybridization steps.

DNA hybridization: A $[^{32}P]$-labeled DNA in a 1x prehybridization mixture was heated for 10 min in boiling water to denature the DNA, then quickly chilled in ice water and poured into a plastic container. The prehybridized membrane was transferred into the mixture and incubated at 42°C overnight with slow shaking. The membrane was rinsed briefly with a small amount of 1x low stringency buffer to remove the excess, unbound probe. The membrane was then washed twice with 1x low stringency buffer at 55°C for 30 min and followed by two washes with 1x high stringency buffer.

Autoradiography: A hybridized and washed membrane was placed between two sheets of plastic wrap in a Kodak X-Omatic® cassette with incorporated intensifier screens (Eastman Kodak Co., Rochester, N.Y.). Working in the darkroom , a piece of Kodak X-OMAT™ AR film was placed on top of the membrane, then the cassette was closed firmly and kept at -80°C. The film was usually exposed about 6 to 12 h and developed.

J. Preparation of competent bacterial cells for electroporation

The procedure developed by Dower et al. (1988) was followed. A 10-ml portion of an overnight culture was transferred into 1 l of L broth and incubated at 30°C with vigorous shaking until the OD at 600 nm reached 0.5 to 0.8. The culture was then chilled on ice for at least 30 min, and the cells were pelleted at 5,000 rpm (in a Sorvall GSA rotor) for 15 min. The pellet was washed three times: first, in 1 l of cold distilled water; next, in 500 ml of cold distilled water, and thirdly, in 20 ml of cold 10% glycerol in distilled water. Finally, the bacterial cells were suspended in 10% glycerol to a final concentration of at least 3 x 10^{10} cells/ml and 40 μl aliquots were dispensed into 1.5-ml microfuge tubes, and stored frozen at -70°C.

K. Transformation of DNA

Three different methods of plasmid transformation have been used in this study.

(1) *The $CaCl_2$ heat-shock method* (Davis et al., 1986): A 40-μl aliquot of a competent bacterial cell suspension (prepared as described above) was suspended in 160 μl of ice-cold 100 mM $CaCl_2$ for 15 min. Plasmid DNA was added to the mixture, left on ice for 3 min, and then placed in a 42°C waterbath for 3 min. An SOC medium (800 μl) was then added to the mixture and incubated at 30°C for 45 min before plating on selective media containing appropriate antibiotic.

(2) *The quick freeze-thaw method* (De Vries et al., 1984): A bacterial suspension in $CaCl_2$ and plasmid DNA were prepared as in $CaCl_2$ heat-shock method. The mixture was frozen in dry ice and acetone for 2 min and then

thawed in a 32°C waterbath for 2 min. This cycle was repeated. An 800-µl volume of SOC medium was added to the mixture and incubated at 30°C for 45 min before plating on selective media containing appropriate antibiotic.

(3) *The electroporation method* (Electro-transformation Manual, Bio-Rad Laboratories, Richmond, Calif.): Plasmid DNA (50-500 ng) was mixed with 40 µl of competent cells and transferred to a cold cuvette and incubated in ice for at least 1 min before electroporation. Electroporation was performed using the Bio-Rad Gene Pulser (Bio-Rad Laboratories) (capacitance, 25 µF; voltage, 2.5 kV; pulse, 200 ohms, as recommended for *E. coli* [Dower et al., 1988]). Immediately following electroporation, 960 µl of SOC medium was added to the cuvette and mixed gently with a Pasteur pipette. The cells were then transferred to a 1.5-ml microfuge tube and incubated at 30°C for 45 min before plating on a selective media containing appropriate antibiotic.

L. P1 bacteriophage preparation and transduction

A high titer of P1 phage lysate was prepared by propagating P1vir in a bacterial strain. A thick-agar plate method similar to the one described in the preparation of λ phage was used. In this case, however, soft agar which contained both 5 mM $CaCl_2$ and 10 mM $MgSO_4$ was utilized (Silhavy et al., 1984).

Transduction was performed following the protocol of Silhavy et al. (1984). A single colony of a recipient strain was grown overnight in 5 ml L broth at 37°C, pelleted by centrifugation at 5,000 rpm (in a Sorvall SS-34 rotor) for 10 min and suspended in 2.5 ml of 10 mM $MgSO_4$ containing 5 mM $CaCl_2$. Mixtures of P1vir plus recipient cells at several different MOI (multiplicity of infection) were set up. After the tubes were incubated at 30°C for 30 min without shaking, 0.1 ml of 1 M sodium citrate was added to each tube to chelate the cations and prevent reinfection of cells by the phage. The mixtures were centrifuged at 10,000 rpm (in a Sorvall SS-34 rotor) for 10 min. The pellet was suspended in 0.1 ml B buffer, spread on an L agar plate containing the appropriate antibiotic and incubated overnight at 37°C.

M. Cloning

Cloning in plasmid vector: A symmetric ligation procedure of Davis et al. (1986) was used to clone a known DNA fragment into a plasmid vector. First, in a 20-μl reaction volume, a 0.1 μg linearized, dephosphorylated pBluescript™ vector (Stratagene, La Jolla, Calif.) was mixed with insert DNA (at a ratio of 1:3) in the presence of ligation buffer and T4 ligase. The mixture was incubated at 14°C overnight, then inactivated at 65°C for 10 min. The ligation mixture was then transformed into competent *E. coli* cells prepared by either $CaCl_2$ heat-shock or electroporation. Transformants grown on an L agar plate containing appropriate antibiotic were screened for the desired clones.

Cloning in λ vector: This procedure was used to clone specific DNA fragments containing *lacZ* from λ*plac*Mu randomly inserted in the chromosome or to clone an extended DNA fragment covering 7.2 kb of pBJ1. The specific DNA fragments were recovered from a gel, following electrophoresis, ~~using~~ Geneclean™ (Bio 101, La Jolla, Calif.). A reaction mixture consisted of 1 μg of LambdaDASH™ (cut with *Bam*HI) (Stratagene) mixed with 0.3 μg of the insert

DNA fragments, 0.5 μl of 10 mM ATP, 0.5 μl of 10x ligation buffer, and 4 Weiss units of T4 ligase. The mixture was incubated for 1 h at room temperature, then further incubated overnight at 14°C. The next day, the ligated λ DNA and insert in the ligation mixture were packaged using the GIGAPACK PLUS™ kit (Stratagene). The packaging process involved mixing 4 μl of the ligated DNA with two different sonic extracts which was prepared from two different λ lysogenic strains. One extract containing preheads was prepared from strain BHB2690 and second extract containing the D protein and other components necessary for packaging was prepared from strain BHB2688. The reaction was performed at room temperature for 2 h. Then, 500 μl of SM buffer and 20 μl of chloroform were added, mixed, and centrifuged to pellet the debris. The supernatant was diluted and plated on *E. coli* P2392 as a host strain. The λ clones containing the desired DNA fragments were screened by a plaque hybridization method.

Quick subcloning: The conventional subcloning technique involves the excision of a particular DNA fragment using restriction enzymes, the purification of the target fragment and its ligation into the compatable ends of a similarly digested vector. In this study, I developed a quick and simple procedure for subcloning DNA fragments originally cloned in a plasmid vector, without isolation of the DNA fragments to be subcloned and without preparation of a plasmid vector.

The DNA to be subcloned was digested with the desired restriction enzymes. This treatment was followed by phenol extraction to remove the restriction enzymes. The digested DNA was then treated with ligase under the appropriate conditions. This process yields several possible combinations of ligated products, which have to be screened by selection for the desired clone.

A plasmid, pBJ1, was used as a sample for this procedure. This plasmid contains a DNA fragment of *K. pneumoniae* cloned in the plasmid vector pBluescript. The restriction map of pBJ1 is shown in Fig. 9. Plasmid pBJ1 DNA was isolated by using the method of Krieg and Melton (Promega Notes, March, 1985) with minor modifications. Approximately 1 μg of the DNA was cut with the restriction endonucleases *Hin*dIII and *Pst*I in reaction buffer 2 (Bethesda Research Laboratories, Inc.) followed by two extractions with phenol:chloroform (1:1) and with chloroform once. The upper aqueous layer was transferred to a fresh tube. One-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol were added and incubated at -80°C for 10 min to precipitate the DNA. The precipitated DNA was pelleted by centrifuging at 4°C for 10 min using an Eppendorf microcentrifuge. After the removal of the supernatant, the DNA pellet was rinsed once with 70% ethanol and vacuum dried. The resulting DNA was then dissolved in 15 μl of TE buffer. A volume of 2 μl of ligation buffer, 2 μl of 10 mM DTT, and 1 μl of T4 ligase (4 Weiss units) were added to the dissolved DNA. The ligation reaction was left to proceed at room temperature for 30 min, then for another 4 h at 14°C. The transformation of this ligated DNA into *E. coli* NM522 was performed by either the $CaCl_2$ heat-shock method or electroporation. White transformant colonies grown on L agar containing X-Gal and IPTG were randomly selected for plasmid DNA preparation by a mini-scale procedure (Ausubel et al., 1987). Finally, the plasmid DNA was cut with *Hin*dIII and *Pst*I, separated by gel electrophoreses (0.8% agarose) and the DNA fragments to be subcloned were determined (Fig. 3).

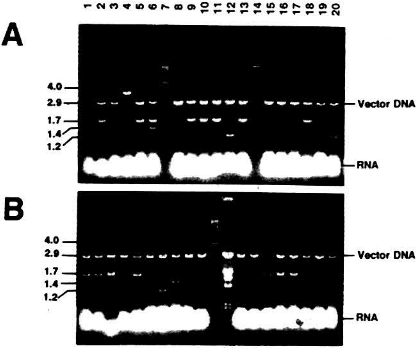
N. Construction of plasmid libraries of *K. pneumoniae* GM236 chromosomal DNA

A 5-µg quantity of pBR322 was cut with 2.5 µl of *Bam*HI (10 unit/µl) in a 20-µl reaction volume for 1 h at 37°C. A small portion of the mixture was tested by mini-gel electrophoresis to confirm that the vector DNA had been completely cut. Linearized pBR322 was dephosphorylated by treatment with calf intestinal alkaline phosphatase for 30 min at 37°C. The mixture was then extracted once with phenol:chloroform (1:1) and once with chloroform only, followed by ethanol precipitation. The DNA pellet was dissolved in 50 µl of TE buffer and stored at -20°C.

Chromosomal DNA of *K. pneumoniae* GM236 ($r^-_{KpnII}m^-_{KpnII}$) purified by the method described above (Section E), was partially digested with *Sau*3A1 endonuclease at 37°C for 15 min (Ausubel et al., 1987). Following agarose gel electrophoresis, DNA fragments between 3 kb and 10 kb were recovered from the gel using a Geneclean™ kit from BIO 101.

A ligation reaction was set up with dephosphorylated *Bam*HI-linearized plasmid pBR322 and the *Sau*3AI genomic DNA fragments. A 25-µl volume of ligation reaction mixture consisted of approximately 1 µg of insert DNA, 0.3 µg of dephosphorylated *Bam*HI-linearized pBR322, 2.5 µl of 10x ligation buffer, 2.5 µl of 10 mM ATP, 1 µl of T4 ligase (4 Weiss units), and distilled water. Ligation

FIG. 3. Photograph of an agarose gel electrophoresis of plasmids extracted from quick subcloning transformants. Plasmids were digested with *Hin*dIII and *Pst*I. Lanes 7, 14 (in the upper set "A") and lane 11 (in the lower set "B") are 1-kb DNA marker, whereas lane 12 (in the set "B") is the plasmid pBJ1 digested with *Hin*dIII and *Pst*I. Numbers on the top indicates lanes, while numbers on the side represent DNA size, in kb.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A
4.0
2.9
1.7
1.4
1.2
Vector DNA
RNA
B
4.0
2.9
1.7
1.4
1.2
Vector DNA
RNA

reactions were performed at 14°C overnight and the reaction was terminated by heating at 65°C in a water bath for 10 min. The mixture was then extracted once with phenol:chloroform (1:1) and once with chloroform only. The DNA in the aqueous phase was transferred to a new tube, one-tenth volume of cold 3 M sodium acetate (pH 5.2) and two volumes of cold absolute ethanol were added and the DNA was precipitated at -20°C for 30 min. The DNA was pelleted by centrifugation for 10 min at 4°C in an Eppendorf microcentrifuge and rinsed twice with 70% ethanol to dissolved excess salt. The sample was dried under vacuum. Finally, the pellet was dissolved in 10 μl of TE buffer and used for electroporation.

O. **Selection of clones expressing restriction endonuclease activity**

A 1-μl portion of the ligated DNA mixture obtained from the above preparation was used to transform competent $r^-_{KpnAI}m^-_{KpnAI}$ *K. pneumoniae* GM236R cells by electroporation. The transformation mixture was plated on 1.5% L agar plates containing ampicillin (1,000 μg/ml) and incubated overnight at 30°C. Transformants were screened for r^+_{KpnAI} recombinant plasmid clones by replica plating on a freshly prepared 1.5% L agar plate supplemented with ampicillin and seeded with approximately 2 x 10^7 non-modified SBS.0 phage. After overnight incubation at 30°C, the surviving colonies were tested for restriction activity by cross streaking. The r^+ clones were then confirmed by a spot test.

P. **DNA labeling by nick translation** (Rigby et al., 1977)

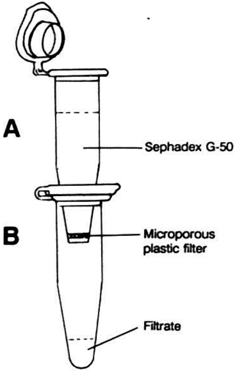
DNA probes were labeled with [α-^{32}P]-dCTP radionucleotide by following the protocol in a commercial nick translation kit (Bethesda Research Laboratories, Inc.). A 5-μl volume of dATP, dGTP, dTTP mixture; 0.5 to 1 μg of DNA to be labeled; 15 μl [α-^{32}P]-dCTP (3,000 Ci/mmol); 5 μl of DNA polymerase/DNase I and distilled water to make a total volume of 50 μl were mixed in a reaction tube and centrifuged briefly in an Eppendorf microcentrifuge. The mixture was then incubated at 15°C for 1 h and the reaction was stopped by adding 5 μl of 0.5 M EDTA.

Unincorporated radionucleotides were fractionated from labeled DNA by passing the labeling mixture through a Sephadex G-50 column. The column was prepared as shown in Fig. 4. The column was precentrifuged for 4 min at 2,500 rpm in an IEC Model HN-SII table-top centrifuge (International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) with a swing bucket to remove excess liquid from the Sephadex. The labeling mixture was loaded on top of the Sephadex column. After centrifugation for 10 min at 2,500 rpm, the eluent was diluted and counted in a liquid scintillation counter. The labeled reaction was diluted to approximately 3 to 5 x 10^6 cpm/ml with 1x prehybridization solution.

Q. **Recovery of DNA from agarose gel using the Geneclean™ kit**

DNA fragments separated by electrophoresis were recovered from agarose gel using the commercially available Geneclean™ kit (Bio 101). DNA fragments were cut from an agarose gel and mashed with a small spatula in a 1.5-ml microfuge tube. A 2.5x volume of saturated NaI was added and the tube was

FIG. 4. Construction of a Sephadex G-50 column used to separate radioactively-labeled DNA from unincoporated radionucleotides. The conical bottom of tube A was removed and a microporous plastic filter (medium pore size) was securely wedged in place as shown. Sephadex G-50 was loaded on top of the plastic filter. An opening was made in the cap of tube B. The tapered end of tube A was inserted into tube B.


A
Sephadex G-50
B
Microporous
plastic filter
Filtrate

placed at 45 to 55°C for 5 min to dissolve the agarose. A 5-μl portion of a Glassmilk™ suspension was added to the mixture, mixed and placed on ice for 5 min to allow binding of DNA to the Glassmilk beads. The DNA-glassmilk complex was pelleted from the mixture by centrifuging for 5 s in an Eppendorf microcentrifuge and the supernatant was discarded. The pellet was washed three times with 0.5 ml of NEW wash solution (as provided in the kit) to eliminate excess NaI. DNA was eluted from the glass beads by adding 5 to 10 μl of TE buffer and incubating at 45 to 55°C for 3 min. After centrifugating for 30 s, the DNA in the supernatant was collected and used.

R. Colony- and plaque-hybridization

Transfer of colonies growing on a plate to a nitrocellulose membrane was performed following the procedure of Davis et al. (1986). A circular, dry nitrocellulose membrane was placed evenly on the plate containing colonies to be screened. After the membrane was completely wet, the edge of the membrane and agar were marked using an 18 gauge needle to later facilitate localization of the colonies of interest. The membrane was then peeled from the plate and placed (colony side up) for 5 min on a filter saturated with 0.5 M NaOH. The membrane was sequentially transferred to a second filter saturated with 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.4) for another 5 min and finally to a third filter soaked with 1.5 M NaCl and 2x SSC for 5 min. After blotting the excess liquid and air drying for 1 h, the membrane was baked for 2 h at 80°C under vacuum. This membrane was used for prehybridization and then hybridization to a $[^{32}\mathrm{P}]$-labeled probe (Southern hybridization).

A similar procedure was used to transfer plaques formed in a bacterial lawn to nitrocellulose membrane (Davis et al., 1986). Except that the plaques were allowed to adsorb to the membrane for 20 min, air dried for 30 min at room temperature and sequentially treated for 1 min intervals with three different solutions: 0.2 M NaOH and 1.5 M NaCl; 2x SSC and Tris-HCl (pH 7.4); and 2x SSC.

S. DNA sequencing

Preparation of plasmid DNA template: DNA sequencing by Sanger's dideoxy termination method (Sanger et al., 1977) followed the protocol of the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio) using $[\alpha\text{-}^{35}\mathrm{S}]$-dATP (New England Nuclear Corp., Boston, Mass.) as the labeled nucleotide. Plasmid DNA (at least 3 μg) was prepared, by an alkaline-SDS lysis method, from 5 ml of an overnight culture grown in an appropriate antibiotic. The plasmid sample was dissolved in 20 μl of TE buffer. An 18-μl volume of the DNA was denatured by mixing with 2 μl of 2 M NaOH at room temperature for 5 min, then 8 μl of 5 M ammonium acetate (pH 7.5) and 100 μl of cold 95% ethanol were added to neutralize and precipitate the denatured DNA. After incubation for 30 min at -20°C, the sample was centrifuged at 15,000 rpm in a microcentrifuge, at 4°C for 30 min. The DNA pellet was washed twice with 500 μl cold 70% ethanol, and dried under vacuum.

Annealing and polymerization reactions: The annealing reaction was then performed by adding 6 μl of sterile distilled water, 2 μl of Sequenase® reaction buffer, and 2 μl of primer to the dried DNA (the DNA was suspended in the aqueous mixture). The mixture was placed in a 65°C water bath for 2 min, then the waterbath was allowed to cool down to room temperature (less than 30°C). Next, 1 μl of DTT (0.1 M), 0.5 μl of $[\alpha\text{-}^{35}\text{S}]$-dATP (1,250 Ci/mmol), 2 μl of 1:5 diluted labeling mix, and 2 μl of 1:8 diluted sequenase enzyme were added and the mixture was incubated at room temperature for 2 to 5 min. Consequently, 3.5-μl aliquots were transferred to a set of four termination reaction tubes containing 2.5 μl(8 μM) of ddGTP, ddATP, ddCTP, and ddTTP, respectively. Finally, 4 μl of stop reaction mix (provided in the kit) were added to each tube to stop the reaction.

Polyacrylamide gel electrophoresis: An 8% acrylamide sequencing gel was prepared as described in the United Biochemical Corporation sequencing handbook. The gel was prerun for 30 min at 55 watts, then 3 μl of preheated sequencing sample mixtures (2 min at 75°C) were loaded and run at a constant power (55 watts) for 3 to 4 h or until the bromophenol blue dye ran off the bottom.

The upper glass plate was removed and the gel, which was still stuck to the lower plate, was carefully placed into fixer (10% methanol:10% acetic acid) for 30 min. The plate with the gel was removed from the fixer and the gel was transferred to a piece of blotting paper. After covering the gel with plastic wrap, the gel was dried under vacuum at 80°C for 45 min. Autoradiography of the gel was performed by exposing the gel overnight to Kodak XAR-5 film (Eastman Kodak Co.) at room temperature.

T. Reproduction of figures

A Bio Image scanner with the Visage 4.6K software (Millipore Corp., Bedford, Mass.) was used to reproduce figures from photographs of agarose gel electrophoresis of DNA and X-ray autoradiographs.

RESULTS

PART I. CHARACTERIZATION OF KpnAI AND KpnBI

A. Differences of KpnAI and KpnBI and other *Klebsiella* systems

In order to confirm that the KpnAI (in *K. pneumoniae* M5a1) and KpnBI (in *K. pneumoniae* GM236) R-M systems were distinct from each other and from known systems in other strains of *Klebsiella* a number of tests were done.

To confirm the distinct identities of KpnAI and KpnBI, the EOP of phage SBS with different modifications were determined on M5a1 and its r⁻ mutant, 5022, and on GM236 and its r⁻ mutant, GM238. The results of these EOP are shown in Table 5. M5a1 (with the R-M system KpnAI) restricted phage propagated on GM236 (with the R-M system KpnBI) and GM238, and GM236 restricted phage propagated on M5a1 and 5022. These results confirmed the different identities of KpnAI and KpnBI.

One type II restriction endonuclease, *Kpn*I, has been isolated from a strain of *K. pneumoniae*, and four type II restriction endonucleases are isoschizomers of other restriction endonucleases found in other strains of *Klebsiella*. These are *Bsp*MII, *Eco*RII, *Bss*HII, and *Pst*I. In order to determine if KpnAI and KpnBI are different from any of these five type II systems, chromosomal DNA from M5a1 and GM236 were digested with each enzyme. If phage DNA from either of these strains is modified by any of these type II systems, the DNA would be resistant to digestion by the corresponding restriction endonucleases. The results of these digestions are shown in Fig. 5.

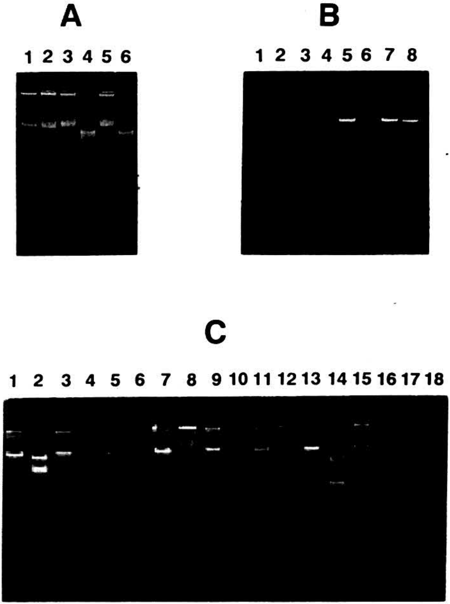
TABLE 5. EOP[a] of SBS phages propagated on different hosts and plated out on four different strains of *K. pneumoniae*

Bacterial strain	R-M phenotype	Efficiency of SBS phage propagated on:[b] 5022	M5a1	GM238	GM236
5022	$r^-_{KpnAI}m^-_{KpnAI}$	1.0	1.0	1.0	1.0
M5a1	$r^+_{KpnAI}m^+_{KpnAI}$	10^{-2}	1.0	10^{-2}	10^{-2}
GM238	$r^-_{KpnBI}m^-_{KpnBI}$	1.0	1.0	1.0	1.0
GM236	$r^+_{KpnBI}m^+_{KpnBI}$	10^{-2}	10^{-2}	10^{-2}	1.0

[a] Semiquantitative restriction test was performed as described in the Methods. Bacteria were grown at 37°C and plates were incubated at 37°C.

[b] Each SBS phage was propagated on a different strain of *K. pneumoniae*. SBS.M5a1 was propagated on strain M5a1, similarly, SBS.5022, SBS.GM236, and SBS.238 were propagated on strains 5022, GM236, and GM238, respectively.

FIG. 5. Agarose gel electrophoresis of chromosomal DNA digested with different restriction enzymes. (A) DNA of *K. pneumoniae* strains OK8 (lanes 1 and 2), M5a1 (lanes 3 and 4), and GM236 (lanes 5 and 6) digested with *Kpn*I restriction endonuclease. Lanes 1, 3, and 5 are genomic DNA without *Kpn*I. Lanes 2, 4, and 6 are genomic DNA with *Kpn*I. (B) DNA of *K. pneumoniae* strains M5a1, GM236, and OK8 digested with *Bsp*MII. Lanes 1,3,5, and 7 are DNA without restriction endonucleases; lanes 2,4,6, and 8 are DNA cut with *Bsp*MII; lanes 1 and 2 are M5a1 DNA; lanes 3 and 4 are GM236 DNA; lanes 5 and 6 are OK8 DNA; lanes 7 and 8 are λ DNA. (C) DNA of *K. pneumoniae* strains M5a1 and GM236 digested with other type II isoschizomers of restriction endonucleases found in *Klebsiella* strains. Lanes 1,2,7,8,13, and 14 are λ DNA; lanes 3,4,9,10,15, and 16 are M5a1 DNA; lanes 5,6,11,12,17, and 18 are GM236 DNA; Lanes 1,3,5,7,9,11,13,15, and 17 are DNA without restriction endonucleases. Lanes 2,4, and 6 are DNA cut with *Bss*HII; lanes 8,10, and 12 are DNA cut with *Eco*RII; lanes 14,16, and 18 are DNA cut with *Pst*I.


A
1 2 3 4 5 6
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Both M5a1 and GM236 DNA were completely digested by all five type II restriction endonucleases. It can therefore be concluded that KpnAI and KpnBI were both different from the R-M systems that code for all presently recognized type II restriction endonucleases.

B. Effect of higher temperature on KpnAI and KpnBI

The efficiencies of restriction of different R-M systems may vary considerably for the same phage indicator. For example, the EOP of unmodified λ phage on *E. coli* with the K or B system is about 10^{-4} (Arber and Linn, 1969), about 10^{-5} with the P1 system (Arber and Linn, 1969), and about 10^{-7} with the LTI system (De Backer and Colson, 1991). Factors that affect the EOP include the number of specificity sites on the DNA and the temperature sensitivities of the modification methylases and restriction endonucleases (Holloway, 1965; Colson and Van Pel, 1974). The effect of temperature on both the restriction and modification activities of KpnAI and KpnBI were tested. The EOP of SBS on GM236 and M5a1 at different temperatures are shown in Table 6.

Cultures of M5a1 and GM236 grown at 30°C, 37°C, and 42°C were used as lawns in three duplicate restriction tests which were then incubated at 30°C, 37°C, and 42°C. SBS.0 was used as the indicator phage. No differences in the EOP of phage SBS.0 plated on M5a1 ($r^{+}_{KpnAI}m^{+}_{KpnAI}$) at the three temperatures were observed (Table 6). However, the EOP varied when phage SBS.0 was plated on GM236 ($r^{+}_{KpnBI}m^{+}_{KpnBI}$) and incubated at 30°C (EOP of 10^{-3}), 37°C (EOP of 10^{-2}), and 42°C (EOP of 1.0). The modification activities of both KpnAI and KpnBI were temperature-insensitive since EOP of 1.0 were obtained when single plaques

TABLE 6. EOP of unmodified SBS phage on *K. pneumoniae* GM236 and M5a1 grown at different temperatures[a]

Bacterial strain	Restriction phenotype	Bacteria grown at[b] 30°C	37°C	42°C
GM238	r^{-}_{KpnBI}	1.0	1.0	1.0
GM236	r^{+}_{KpnBI}	10^{-3}	10^{-2}	1.0
5022	r^{-}_{KpnAI}	1.0	1.0	1.0
M5a1	r^{+}_{KpnAI}	10^{-3}	10^{-3}	10^{-3}

[a] Phage SBS grown in *K. pneumoniae* GM238 (unmodified phage, SBS.0) and in *K. pneumoniae* GM236 (modified phage, SBS.KpnBI) were used for the semiquantitative restriction test as described in the Methods. SBS.KpnBI resulted in an EOP of 1.0 on both strains. EOP of SBS.0 on strain GM236 grown at different temperatures relative to GM238 (EOP of 1.0) are shown. Similarly, phage SBS.0 grown in *K. pneumoniae* 5022 and SBS.M5a1 grown in *K. pneumoniae* M5a1 were used for testing in KpnAI system and EOP of strain M5a1 grown at different temperatures relative to strain 5022 (EOP of 1.0) are also shown.

[b] Bacteria were grown overnight at 30°C, 37°C, and 42°C. After plating, the plates were incubated at temperature at which the strains were grown.

of SBS on both strains were grown at each of the temperatures and tested for modification. These results indicate that only the restriction activity of KpnBI is temperature-sensitive.

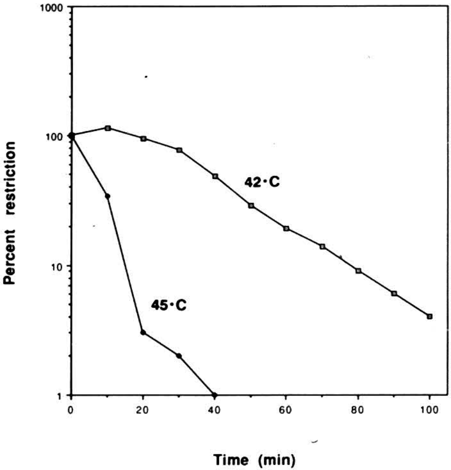
In order to determine if the higher temperature primarily affects expression of the genes or if its effect is to inactivate the protein product of the genes the following experiment was conducted. Cells grown at 30°C were collected at mid-log phase, washed, suspended in B buffer, and incubated at 42°C and 45°C. Bacterial samples were collected and quantitative restriction activities were determined at 10-min intervals for 100 min. Results of the degree of restriction were plotted against time (Fig. 6).

If the enzyme were heat-labile, the restriction activity would be expected to decrease immediately, whereas, if the enzyme expression were heat-labile, the cell would be expected to retain the restriction activity until it is diluted out by cell division. At 42°C the restriction activity of the bacteria decreased more than 95% in 90 min. At 45°C, the restriction activity decreased even more rapidly and was undetectable within 40 min. These results suggested that the KpnBI system was sensitive to elevated temperature at the protein level and probably not at the transcriptional level. That is to say, the activity of the *Kpn*BI restriction endonuclease was sensitive to elevated temperatures.

C. Effect of glycerol on restriction activities of KpnAI and KpnBI

During the course of experiments to increase the efficiency of transformation by the use of glycerol (Stuy and Walter, 1986), glycerol was observed to have an effect on the restriction activity of KpnBI. Overnight

FIG. 6. Effect of temperature at 42°C and 45°C on *Kpn*BI restriction endonuclease activity. The restriction efficiency at zero time was considered to be 100% restriction.


1000
100
10
1
Percent restriction
0
20
40
60
80
100
Time (min)
42·C
45·C

cultures of GM236 (KpnBI) grown in L broth containing 10% glycerol had no detectable restriction activity. The EOP of SBS phage on GM236 and M5a1 grown in 10% glycerol are shown in Table 7. This unusual observation was named the "glycerol effect". By contrast, the restriction activity of M5a1 (KpnAI) grown under similar conditions was not affected by 10% glycerol. Thus, these two *Klebsiella* R-M systems were distinctive with aspect to their function in the presence of glycerol.

The concentration of glycerol and the duration of glycerol treatment required to affect the restriction activity of KpnBI were evaluated. An overnight culture of GM236 grown at 30°C was subcultured into L broth containing different concentrations of glycerol. Growth was followed by monitoring the OD_{600}. Restriction activities were examined at 2-h intervals for 10 h. The EOP of SBS phage on GM236 in different concentrations of glycerol plotted against time of incubation are shown in Fig. 7A.

The EOP of unmodified phage SBS increased from about 10^{-3} without glycerol to about 10^{-1} at 10% glycerol. The rate of growth of GM236 at different concentrations of glycerol is shown in Fig. 7B. There was little effect on the rate of growth, except at a highest glycerol concentration, 10%.

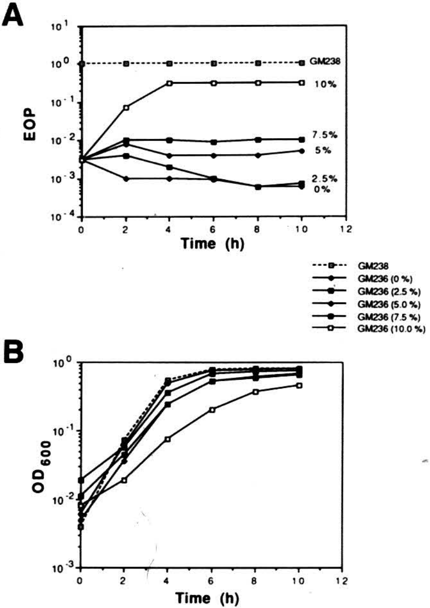
This observation that restriction activity is reduced when the bacteria are grown in media containing glycerol is unique and does not appear to have been reported for any other R-M system.

TABLE 7. EOP of unmodified SBS phage on *K. pneumoniae* GM236 and M5a1 grown overnight in L broth containing 10% glycerol

Bacterial	Incubation time (h)[a]				
strain	0	1	3	6	Overnight
GM238	1.0	1.0	1.0	1.0	1.0
GM236	8 x 10^{-3}	4 x 10^{-3}	2 x 10^{-3}	1 x 10^{-3}	2 x 10^{-3}
GM236 + glycerol	2 x 10^{-2}	2 x 10^{-2}	3 x 10^{-1}	1.5 x 10^{-1}	1.0
5022	1.0	ND	ND	ND	1.0
M5a1	1 x 10^{-3}	ND	ND	ND	1 x 10^{-3}
M5a1 + glycerol	1 x 10^{-3}	ND	ND	ND	1 x 10^{-3}

[a] The cultures were incubated at 30°C. At specified time intervals, the cultures were tested for restriction activity. The EOP of SBS.0 on GM236 and M5a1 relative to GM238 and 5022, respectively, are shown. GM238 and 5022 were the nonrestricting controls. ND, not done.

FIG. 7. Effects of glycerol on restriction activity and on growth of *K. pneumoniae* GM236. (A) The EOP of SBS.0 on GM236 grown in various concentrations of glycerol (0 to 10%) in L broth. (B) The $OD_{[illegible]}$ of GM236 grown in various concentrations of glycerol. Bacteria were grown overnight to late-stationary phase and subcultured into L broth and L broth containing different concentrations of glycerol (2.5, 5, 7.5 and 10%). The cultures were incubated at 30°C with rotation. Optical density reading ($OD_{[illegible]}$) and quantitative restriction tests were performed at time zero and every 2 h for 10 h.


A
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
EOP
0
2
4
6
8
10
12
Time (h)
GM238
10%
7.5%
5%
2.5%
0%
GM238
GM236 (0 %)
GM236 (2.5 %)
GM236 (5.0 %)
GM236 (7.5 %)
GM236 (10.0 %)
B
10^0
10^{-1}
10^{-2}
10^{-3}
OD_{600}
0
2
4
6
8
10
12
Time (h)

D. Distribution of transposon-induced restriction and modification mutants of KpnAI and KpnBI

The phenotypes of typical point mutants in restriction-modification systems are of two types, r^-m^+ and r^-m^-. Equal numbers of these two mutant types is a distinctive property of type I and type III systems. In order to investigate if KpnAI and KpnBI might be representatives of either of these two systems, the numbers of the two different mutant types following mutagenesis by operon fusion with λ*plac*Mu was determined for both KpnAI and KpnBI.

Of the 900 GM236 colonies examined, only one spontaneous mutant was detected and this was shown to be r^-m^-.

Transposon mutagenesis with λ*plac*Mu is a widely used method to derive mutants of *E. coli*, but cannot be used for *K. pneumoniae* since phage λ is unable to adsorb to *K. pneumoniae*. However, if *K. pneumoniae* contains the plasmid pTroy11 which encodes (*lamB*) the gene for the λ receptor protein, it can then be infected with λ. Accordingly, pTroy11 was transformed into *K. pneumoniae* M5a1 and GM236 by electroporation.

K. pneumoniae M5a1 with pTroy11 and GM236 with pTroy11 were infected with λ*plac*Mu. Pools containing random λ*plac*Mu lysogens were collected. Conjugation of these pools with *E. coli* 1228F'JR2 as the donor were performed to allow the enrichment of R-M mutants. Conjugants were then screened for their R-M phenotype. Seven restriction mutants of M5a1 were obtained, 3 of which (43%) were ~~r^-m^+~~ and 4 were r^-m^- (57%). There were 38 r^- mutants of GM236, 14 of which (37%) were r^-m^+ and 24 (63%) were r^-m^-. The isolation of both r^-m^+ and r^-m^- mutants at the same time strongly suggests that

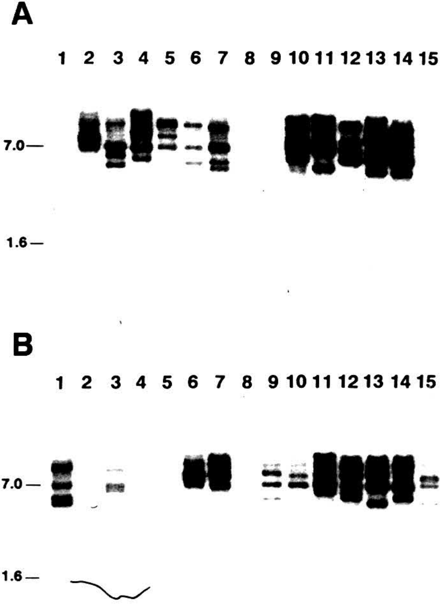
KpnAI and KpnBI are members of type I or type III systems. It is highly improbable that they are type II systems.

There is an *Eco*RI site within the *lacZ* segment of the λ*plac*Mu transposon (O'Connor and Malamy, 1983). Therefore, DNA hybridization of *Eco*RI-digested *K. pneumoniae* mutant chromosomal DNA with the *lacZ* probe would select for mutations adjacent to the transposon. Thus, a single insertion would yield a single hybridization band in Southern hybridizations when *lacZ* is used as a probe. Multiple bands would indicate multiple insertion sites. Southern hybridization of *lacZ* to *Eco*RI-digested DNA of all the restriction mutants of *K. pneumoniae* M5a1 and GM236 showed multiple bands. Representative results of these hybridizations are shown in Fig. 8. Consequently, all of these mutants had multiple λ*plac*Mu insertions.

To obtain single insertion R-M mutants, further transposon-induced restriction mutants of GM236 were done under different conditions as shown in Table 8. The presence of Ca^{2+} and Mg^{2+} ions yielded a significantly higher number of mutants. Washing the host cells twice with sterile distilled water and increasing the helper phage concentration also yielded more Kan^r mutants.

Eighty-nine restriction mutants of GM236 were obtained, 61 (68.5 %) were r^-m^- and 28 were r^-m^+ (31.5 %). The number of *lacZ* (λ*plac*Mu) insertions in these mutants were determined by DNA hybridization. Six single λ*plac*Mu insertion mutants were obtained, four were r^-m^- and two were r^-m^+. This result again supported the conclusion that the KpnBI R-M system in GM236 was type I or type III.

FIG. 8. Autoradiography showing hybridization of M13 (*lacZ*) with *K. pneumoniae* GM236 λ*plac*Mu mutants with the r^- phenotype. Genomic DNA from the mutants were digested with *Eco*RI and probed with [^{32}P]-M13 (*lacZ*). Numbers on the top indicate lanes, while numbers on the side represent DNA size markers in kb. Panel A (lanes 1-7, and 9-14) and panel B (lanes 1-7, and 9-15) are DNA of GM236 mutants; lane 15 (panel A) is GM236 wild-type DNA; lanes 8 of both panels are 1-kb DNA markers.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
7.0
1.6
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
7.0
1.6

In order to confirm that the single insertion transposons were located within the *hsd* genes, P1 transductions of Kan^r from r^-m^- and r^-m^+ mutants of KpnBI to the recipients, GM236R (r^-m^+) and GM238 (r^-m^-) were conducted. If the transposon were located within the KpnBI, Kan^r recipients would be expected to have the R-M phenotype of the mutant donor. However, all Kan^r transductants retained the recipient R-M phenotype.

In conclusion, a total of 7 r^- mutants (3 r^-m^+, 4 r^-m^-) from the KpnAI system and 127 r^- mutants (42 r^-m^+, 85 r^-m^-) from the KpnBI system were obtained. The occurrence of r^-m^+ and r^-m^- mutants in either system was approximately equal. These results suggest that KpnAI and KpnBI are members of either type I or type III systems. P1 transductions were performed in order to localize the λ*plac*Mu inserted within *hsd*KpnBI region. Although the frequency of spontaneous mutants in the *hsd*KpnBI genes of *K. pneumoniae* GM236 was high (1 in 900), P1 transduction of the λ*plac*Mu fusions to these genes was inefficient. A very low number of Kan^r transductants was obtained and all showed the phenotype of the recipient strain. There are at least two explanations for these results: either λ*plac*Mu failed to insert into the *hsd* genes or they may have become relocated through "Mu transposition".

E. Plasmid transformation in *K. pneumoniae*

a. Efficiency of plasmid transformation in *K. pneumoniae* by different methods

Since transformation of DNA is a necessary step in the isolation of cloned DNA, various methods for transformation were examined.

TABLE 8. Summary of the number of *K. pneumoniae* GM236 mutants per ml obtained using λ*plac*Mu as a mutator under different conditions

Expt	Wash with dH_2O[a]	Cation added[b]	Ratio of λMu: λhelper[c]	# of Kan^r (average/ml)[d]	Total r^-[e]	#r^-m^+[f]	#r^-m^-[f]	# of single insertion
1	No	Mg^{2+}	None	0	0	0	0	0
2	No	Mg^{2+}	1:2	230	12	8	4	1
3	Yes	Mg^{2+}	1:2	1250	12	4	8	1
4	Yes	Mg^{2+},Ca^{2+}	1:1	1870	16	4	12	2
5	Yes	Mg^{2+},Ca^{2+}	1:2	3520	21	3	18	1
6	Yes	Mg^{2+},Ca^{2+}	1:4	7580	28	9	19	1
				Total	89	28	61	6

[a] Bacterial cells were washed twice with sterile distilled water (dH_2O) and then suspended in the original volume of L broth.

[b] $MgSO_4$ and/or $CaCl_2$ was added to a final concentration of 10 mM and 5 mM, respectively.

[c] λMu is λ*plac*Mu53, while λhelper is λ*plac*Mu507 (see Table 4).

[d] These represent the number of λ*plac*Mu insertion mutants in the pool prior to further selection by conjugation with *E. coli* 1228 F'JR2.

[f] Number of mutants obtained after conjugation of the λ*plac*Mu insertion pools in each experiment with *E. coli* 1228 F'JR2.

The number of r^- mutants from each experiment is not equal.

The efficiencies of transformation of pBR322 DNA by (i) $CaCl_2$ heat-shock, (ii) $CaCl_2$ freeze-thaw cycle, and (iii) electroporation were determined. To maximize transformation efficiency, the two restriction-deficient strains of *K. pneumoniae* (5022 and GM238) were used as recipients. The transformation efficiencies (number of transformants per μg of plasmid DNA) of the three methods with *Klebsiella* 5022 and GM236 are shown in Table 9. Transformation efficiency by electroporation was about 10^8 transformants per μg DNA in r^- strains, which was 10^4-fold higher than the efficiencies observed by either heat-shock or freeze-thaw. Thus, it is concluded that electroporation is the most efficient transformation method for *K. pneumoniae* and was the method routinely used.

b. Effect of *Kpn*AI and *Kpn*BI restriction on plasmid transformation

The efficiencies of transformation of different plasmid DNA to the strains carrying KpnAI and KpnBI relative to the r^- mutants of both systems were determined. For this purpose, different plasmids were isolated from bacteria other than *K. pneumoniae*, ranging from 4.3 kb to 15.3 kb, and transformed into r^- and r^+ strains of KpnAI and KpnBI. An appropriate antibiotic resistant marker on the plasmid was used for selection of the transformants. Transformation efficiencies of various plasmid DNA in r^+ and r^- strains of KpnAI and KpnBI are shown in Table 10. The transformation efficiencies in r^+ recipients were 20- to 100-fold less when compared with r^- recipients. The reduction in transformation efficiency is undoubtly due to the restriction activity in r^+ strains. Thus plasmids isolated from the surviving transformants should be modified by the KpnAI or

TABLE 9. A comparison of the efficiency of three methods[a] of pBR322 plasmid transformation in *K. pneumoniae* strains

Bacteria	Restriction phenotype	Transformants (Ap^r colonies/μg DNA)		
		CaCl$_2$ Heat-shock	CaCl$_2$ Freeze-thaw	Electroporation
5022	r^-_{KpnAI}	7.0 x 10^3	1.0 x 10^4	1.0 x 10^6
GM238	r^-_{KpnBI}	4.0 x 10^2	1.0 x 10^3	5.0 x 10^7

[a] The details of these methods are described in Methods.

KpnBI modification system and should therefore fail to be restricted when transformed back to the r^+ host. For example, plasmid pBg6 (isolated from *E. coli*), which exhibited an approximately 100-fold reduction in transformation efficiency (Table 10), was isolated from the surviving transformant strain of M5a1 ($r^+_{KpnAI}m^+_{KpnAI}$) and GM236 ($r^+_{KpnBI}m^+_{KpnBI}$). These modified plasmids were transformed back into M5a1 and 5022 (for the KpnAI system), and into GM236 and GM238 (for the KpnBI system). Transformation efficiencies of modified and unmodified plasmid pBg6 with r^+ and r^- strains of KpnAI and KpnBI are shown in Table 11. As expected, and in contrast to the results of the first round of transformation, approximately equal numbers of transformants were obtained from both the r^+ and r^- strains of both KpnAI and KpnBI systems. It was concluded that pBg6 contained recognition sites for both KpnAI and KpnBI and that the restriction of the plasmids was prevented by using a plasmid modified by the respective system. It was possible that those plasmids, such as pBR322, pTroy11, and pBJ1, which gave about equal transformation efficiencies in both r^+ and r^- recipients, contained no recognition sequences for either system.

As described above, the KpnBI restriction system is temperature-sensitive and growth of the bacteria overnight at 42°C completely abolished the restriction function (Table 6). It is reasonable to assume that the restriction of plasmid DNA, with different modification specificities, can be prevented by simply growing the wild-type ($r^+_{KpnBI}m^+_{KpnBI}$) recipient cells at 42°C. For example, two plasmids, pBg3 and pBg6, were used to transform cells which had been grown overnight at 42°C. Similar transformation efficiencies were observed for both r^- and r^+ recipients as shown in Table 12. Therefore, it was concluded that with the

TABLE 10. Transformation efficiencies of different plasmids isolated from non-*Klebsiella* sources on r^+ and r^- strains of KpnAI and KpnBI, expressed as the number of Ap^r colonies per μg of plasmid DNA

	Plasmid (length, in kb)						
Bacteria (phenotype)	pBR322 (4.3)	pTROY11 (6.3)	pBJ1 (10.1)	pRH1 (10.1)	pBg3 (12.5)	pBg6 (14)	pXC1 (15.3)
5022 (r^-_{KpnAI})	1×10^8	3×10^6	9×10^6	6×10^6	2×10^6	7×10^6	6×10^6
M5a1 (r^+_{KpnAI})	2×10^8	4×10^5	8×10^6	6×10^4	7×10^4	9×10^4	3×10^5
GM238 (r^-_{KpnBI})	2×10^8	3×10^7	1×10^6	3×10^5	3×10^4	3×10^5	4×10^4
GM236 (r^+_{KpnBI})	3×10^8	4×10^7	2×10^6	1×10^5	1×10^4	4×10^3	2×10^3

TABLE 11. Transformation efficiencies of modified plasmids, expressed as the number of Ap' colonies per μg of plasmid DNA

Bacteria	Restriction phenotype	Non-modified pBg6[a]	Modified pBg6[b]
5022	r_{KpnAI}	5×10^4	5×10^5
M5a1	r^{-}_{KpnAI}	1×10^5	4×10^5
GM238	r_{KpnBI}	3×10^3	2×10^3
GM236	r^{-}_{KpnBI}	4×10^3	2×10^5

[a] Non-modified plasmids were obtained from *E. coli* NM522.

[b] Modified plasmids for KpnAI and KpnBI systems were prepared from *K. pneumoniae* strains M5a1 and GM236, respectively.

TABLE 12. Transformation efficiencies of pBg3 and pBg6 in KpnBI strains after bacterial growth at 42°C*, expressed as the number of Ap^r per μg of DNA

	Restriction	Transformation efficiencies	
Bacteria	phenotype.	pBg3	pBg6
GM238	r_{KpnBI}	4×10^6	7×10^6
GM236	r^{-}_{KpnBI}	4×10^6	3×10^6

* Overnight cultures of *K. pneumoniae* GM236 and GM238 at 42°C were used as competent cells and transformation was performed by the electroporation method.

KpnBI system, the restriction barrier can be circumvented by growing the recipient cells at 42°C.

PART II. CLONING OF KpnAI AND KpnBI

Several approaches were used to attempt to clone the *hsd*Kpn genes. These were: A, cloning into phage λ; B, DNA hybridization to other known R-M probes; C, transposon mutagenesis (λp*lac*Mu); and D, cloning into a plasmid.

A. Cloning into phage λ

This method is based on the assumption that a λ phage containing an intact KpnBI modification gene would be capable of modifying its own DNA, enabling it to propagate freely (without restriction) in an r^+ strain, whereas those λ phages without a KpnBI modification gene would be unmodified and therefore would be restricted by the r^+ strain.

A *Bam*HI library of *K. pneumoniae* M5a1 or GM236 chromosomal DNA was ligated to λDASH™, packaged and used to infect *E. coli* LE392 ($r_K m_K$). In order to enrich for m^+ clones, this amplified M5a1 library was used to infect M5a1 containing pTroy11. The amplified GM236 library was used to infect GM236 containing pTroy11. However, λ phage failed to propagate in these strains. Although λ phage produced an area of lysis when a high concentration of the lysate was spotted onto a top layer of *K. pneumoniae* with pTroy11, no plaques were produced at higher dilutions. This result is reminiscent of that obtained with colicins, in which undeveloped phages failed to mature (Bradley, 1967). Thus, although λ phage could infect strains of *Klebsiella* containing pTroy11, the phage failed to propagate to maturity. Therefore this method was unsuitable for the cloning of *Klebsiella* R-M genes.

B. Hybridization with other R-M DNA probes

DNA hybridization can be used as a method for identification of a gene in restriction digests of chromosomal DNA if the gene has reasonable homology with a suitable DNA probe. In this way, R-M genes of the E system of *E. coli* (Fuller-Pace et al., 1985) and genes of the CfrI system of *Citrobacter freundii* (Daniel et al., 1988) were successfully cloned.

Hybridization studies of representative clones of both known type I and type III R-M genes were used as probes in attempts to locate DNA sequences on chromosomal DNA of M5a1 and GM236 that shared homology with these systems. The probes used were specific, chromosomally encoded type I R-M genes *hsd*K, *hsd*A, *hsd*SB, the plasmid-encoded type I R-M gene Eco124/3, and the type III R-M systems of P1, P15, and LTI.

Each probe was hybridized to *Eco*RI-digested chromosomal DNA of both M5a1 and GM236. Results of the hybridizations are summarized in Table 13. Figure 9 shows the hybridization results of the SB probe to *Eco*RI-digested M5a1 and GM236 chromosomal DNA. Two of the probes, SB and K, hybridized to an approximately 7.2-kb fragment of the *Eco*RI-cut GM236 DNA. No probe hybridized to M5a1 DNA. These results were interpreted to indicate that KpnBI probably shared some DNA homology with SB and K, but that KpnAI shared no homology to any of the R-M systems represented by the DNA probes.

TABLE 13. Hybridization of chromosomal DNA of *K. pneumoniae* M5a1 and GM236 with different *hsd* DNA probes

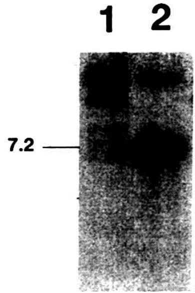
Bacteria (phenotype)	[^{32}P]-Labeled Probe[a]						
	SB	K	A	P1	P15	LTI	Eco124/3
M5a1(KpnAI)	-	-	-	-	-	-	-
GM236(KpnBI)	+	±	-	-	-	-	-

[a] The probes used were:

SB = The 2.6-kb *Hin*dIII-*Eco*RI SB-specific fragment in pXC1 (Ryu et al., 1988).
K = The 3.3-kb *Bam*HI-*Hin*dIII K-specific fragment cloned in pRH1 (Daniel et al., 1988).
A = The 1.1-kb *Eco*RI-*Bam*HI A-specific fragment cloned in pFFP20 (Daniel et al., 1988).
P1 = The 9.4-kb *Bam*HI-*Bam*HI P1-specific fragment in pRH212 (Mural et al., 1979).
P15 = The 9.4-kb *Bam*HI-*Bam*HI P15-specific fragment in pSHI1180 (Bachi and Arber, 1979).
LTI = The 7.4-kb *Pst*I-*Sal*I LTI-specific fragment in pRUCL521 (De Backer and Colson, 1991).
Eco124/3 = The 14.2-Kb *Bam*HI-*Hin*dIII Eco124/3-specific in pUNG30 (Firman et al., 1985).

\+ = Good hybridization
± = Weak hybridization

FIG. 9. Autoradiography of DNA hybridization of a [^{32}P]-labeled 2.6-kb *Hin*dIII-*Eco*RI fragment of pXC1 (*hsdM* clone of SB) to *Eco*RI-digested *K. pneumoniae* M5a1 (lane 1) and GM236 (lane 2) genomic DNA. Numbers on the side represent DNA size in kb, while numbers on the top indicate lanes.


1
2
7.2

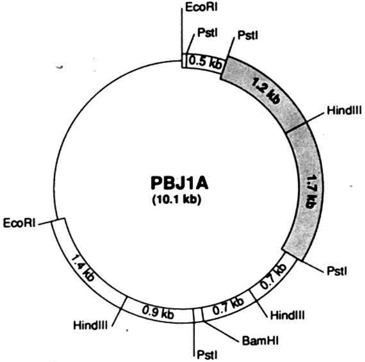
The SB and the K systems belong to the same family (type I) and share DNA homology (Murray et al., 1982). The *hsdR* and the *hsdM* gene products from the SB and K systems functionally complement each other (Van Pel and Colson, 1974). That KpnBI hybridized to both SB and K probes was, therefore, not unexpected.

In order to clone the 7.2-kb *Eco*RI fragment of GM236, the chromosomal DNA was isolated, digested with *Eco*RI, and electrophoresed. DNA fragments covering the 7.2-kb region were recovered, ligated into pBluescript, and transformed into *E. coli* strain NM522 ($r^-_K m^+_K$). Colony hybridization with the SB probe was used to screen for the clones containing the 7.2-kb fragment.

Plasmids were isolated from selected clones which hybridized to the SB probe. Isolated plasmids were purified and subjected to restriction endonuclease digestion. After analyzing the complete, partial, and double restriction endonuclease digestion patterns, a restriction map for the 7.2-kb clone named pBJ1A was constructed and shown in Fig. 10. A clone which contained a different orientation of the 7.2-kb DNA fragment was also obtained and named pBJ1B.

The restriction activity expressed by transformants carrying pBJ1A and pBJ1B was determined with phage λ. However, *E. coli* carrying either pBJ1A or pBJ1B expressed no restriction. Accordingly, both pBJ1A and pBJ1B plasmids were transformed into *K. pneumoniae* GM238 ($r^-_{KpnBI} m^-_{KpnBI}$) in order to examine the R-M phenotypes with phage SBS. However, the *Klebsiella* recipients again expressed neither restriction nor modification. Thus the 7.2-kb fragment either contained only part of the KpnBI genes or was a different gene.

FIG. 10. Restriction map of pBJ1A which is pBluescript™ with a 7.2-kb *Eco*RI-fragment of *K. pneumoniae* GM236 DNA. The SB homologous sequence was located in the section comprised of an approximately 1.7-kb *Pvu*II fragment which overlapped the *Hin*dIII site of 1.2-kb and 1.7-kb *Hin*dIII-*Pst*I fragments (gray zone).


EcoRI
PstI
PstI
0.5 kb
1.2 kb
HindIII
PBJ1A
(10.1 kb)
1.7 kb
EcoRI
PstI
1.4 kb
0.7 kb
HindIII
0.7 kb
0.9 kb
HindIII
BamHI
PstI

Further DNA hybridization experiments were performed to localize the *hsd*SB homologous region on the 7.2-kb *Eco*RI fragment. The 7.2-kb *Eco*RI fragment was cut with different restriction enzymes and then probed with the 2.6-kb *Hin*dIII-*Eco*RI SB fragment. The 2.6-kb *hsd*SB homologous region was located in a 1.7-kb *Pvu*II fragment of pBJ1A, which is indicated in the plasmid map in Fig. 10. When this 1.7-kb fragment itself was used as a probe to hybridize with *Bam*HI-cut pRH1 (an *hsd*K clone) and *Hin*dIII-*Eco*RI-cut pXC1 (an *hsd*SB clone), it hybridized to a 3.3-kb fragment of pRH1 and a 2.6-kb fragment of pXC1 (Fig. 11), verifying that the *Klebsiella* GM236 7.2 kb-fragment shared homology to short regions within both *hsd*SB and *hsd*K.

Accordingly, two fragments - a 1.2-kb *Pst*I-*Hin*dIII fragment and a 1.7-kb *Hin*dIII-*Pst*I fragment, which together include the 1.7-kb *Pvu*II *Klebsiella* fragment - were subcloned from pBJ1A to pBluescript by the "quick subcloning method" (see Materials and Methods) and designated pBJ2 (for the 1.2-kb fragment) and pBJ3 (for the 1.7-kb fragment). The restriction maps of pBJ2 and pBJ3 are shown in Fig. 12. The DNA of both pBJ2 and pBJ3 were sequenced in both directions by the dideoxy termination method for about 300 to 400 bp from the *Hin*dIII site which is located in the central region of the 1.7-kb *Pvu*II *Klebsiella* fragment. A sequence of 672 bp, 40% coverage of the 1.7-kb *Pvu*II fragment, was obtained (Fig. 13).

A computer search for sequence similarity between the 672 bases of pBJ1 and the sequences of *hsd*K was conducted by the method of Myers and Miller (1988) using the NALIGN program version 1.10 of PC-Gene (IntelliGenetics, Mountain View, Calif.). The parameters for sequence comparisons were varied.

FIG. 11. Hybridization analyses and restriction maps of pRH1 and pXC1. (A) Autoradiograph showing hybridization of the 2.6-kb *Hin*dIII-*Eco*RI fragment of *hsd*SB from pXC1 (lane 2) and 3.3-kb *Bam*HI fragment of *hsd*K from pRH1 (lane 1) with [^{32}P]-1.7-kb *Pvu*II fragment of GM236 derived from pBJ1. (B) Restriction maps of the *hsd*K clone (pRH1) and the *hsd*SB clone (pXC1).

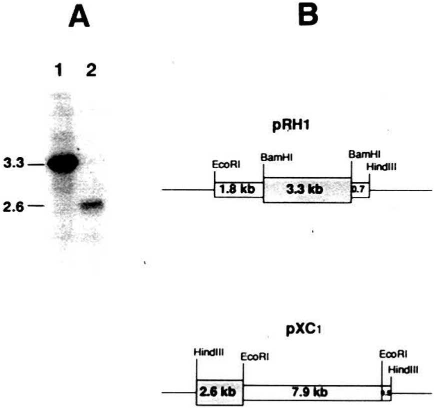
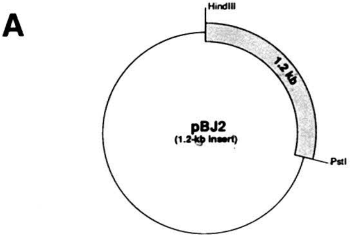

A
B
1
2
3.3
2.6
pRH1
EcoRI
BamHI
BamHI
HindIII
1.8 kb
3.3 kb
0.7
pXC1
HindIII
EcoRI
EcoRI
HindIII
2.6 kb
7.9 kb

FIG. 12. Restriction maps of (A) pBJ2 and (B) pBJ3. pBJ2 and pBJ3 were derived from pBJ1 which covered the SB homologous sequence. pBJ2 contains a 1.2-kb *Hin*dIII-*Pst*I fragment of pBJ1, while pBJ3 contains the 1.7 kb of *Hin*dIII-*Pst*I fragment.


A
HindIII
1.2 kb
pBJ2
(1.2-kb insert)
PstI

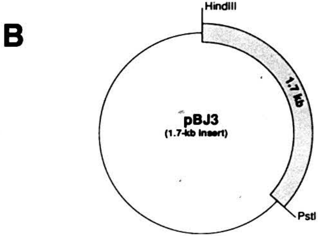

B
HindIII
1.7 kb
pBJ3
(1.7-kb insert)
PstI

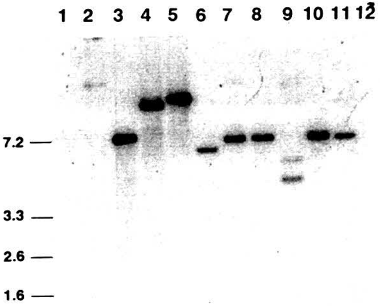
FIG. 13. Nucleotide sequence of the 672 bp covering a portion of DNA in pBJ2 (374 bp) and pBJ3 (298 bp) extended from both sides of the *Hin*dIII site (underlining with boldface).

```
  1 TCGTACGCCT GCTGTTCAAT ACATTGCGAA ATGCCACATG  40
 41 CCGGTGAAAA GTATGACCAT CAGTGATGGC GAAGCGGGTT  80
 81 ATTACGCACC GTTCGCGAAC GGCTGGAAAA CCCACGGCCG 120
121 GGGGTTAAGG TGAGTCTGGA TGACCTATGA ACTGGAGTTT 160
161 GATCCACGAG CCTGGCGCGA ATGGCAGAAG CCTGGCGAGA 200
201 CGGTCAAAAA ACAGTTCAAA AATAAGCTCC AGCAGATTGT 240
241 GCAGAATCCG CGAATTGAGT CGACCAGGCT GAGCGATTTA 280
281 CCGGATTGCT ACAAAATCAA GCTTAAGGCG TCAGGTTATC 320
321 GGTTGGTGTA TCAAGTACGA GATAGTGTGG TGGTGGTTTA 360
361 CGTTATTGCC ATTGGCAAAA GAGAGAAAGC GGCCGTTTAT 400
401 CATCAGGCGA ATAAACGGCT CTAAACGCTA TCGCAAATGA 440
441 TGTACGACCT GGTTGCTGCT GCCGCGCCAG ATAAGCGCCG 480
481 GATCTTTCAA ATCCTGCACA AACTTACCGT CAACCAGCAC 520
521 GTTAATCAGG TTCACGACTT CCATCTGCTC GGCGTTCAGT 560
561 TCGTCAATCT TATAGCCCGT CCAGACCCAG ATATCTTTGC 600
601 CGGGGCATTC GGCGCGTACT GGAACCAGCT TTAAAATATC 640
641 CGCGACGTTC TGCGATCCGG ATCGCCGGAG AG         672
```

The largest continuous region of homology was only 9 bases. Overall sequence similarity was not significant. An open reading frame of 282 bp was detected within the 672-bp region of pBJ1 which showed similarity to the K system. This open reading frame was shown to code for 94 amino acids and had a 98% probability of coding for a protein. Using the same program, significant similarity between *hsd*B and *hsd*K was demonstrated. No significant similarity was detected between this 672-bp segment and any other procaryotic DNA sequences in the procaryotic DNA data bank (which contained 6,854 data sequences). Thus the reason for the homology between the 1.7-kb *Klebsiella* fragment and *hsd*SB and *hsd*K is unclear. It is possible that there is greater similarity within the 60% of the fragment not yet sequenced.

To determine whether the 1.7 kb-*Pvu*II *Klebsiella* fragment consisted of DNA unique to *K. pneumoniae* GM236, the fragment was hybridized to *Eco*RI-digested chromosomal DNA from different enteric bacteria - *E. coli* CSH50 (with *hsd*K), *E. coli* C, *E. coli* 2370 (with *hsd*A), *E. coli* 4001 (with *hsd*SB), *S. typhimurium* LT2 (with *hsd*SA, *hsd*SB, and *hsd*LTI), *K. pneumoniae* M5a1 (with *hsd*KpnAI), and *K. pneumoniae* OK8 (with *hsd*KpnI). This DNA fragment hybridized to all samples of DNA as shown in Fig. 14. Thus the *Klebsiella* fragment probably contains a sequence that is commonly present in most enteric bacteria. This interpretation would suggest that the hybridization of the 2.6-kb SB-specific fragment was associated with a short region of homology which may be unrelated to DNA restriction or modification. *E. coli* C which has no R-M system also showed hybridization to the 1.7-kb *Pvu*II fragment.

FIG. 14. Autoradiograph showing hybridization of the [^{32}P]-1.7-kb *Pvu*II fragment of pBJ1 with chromosomal DNA derived from different enteric bacteria. Genomic DNA were digested with *Eco*RI and probed with the 1.7-kb *Pvu*II fragment. Numbers on the top indicate lanes, while numbers on the side represent DNA size markers in kb. Lane 1, 1-kb ladder; lane 2, *K. pneumoniae* M5a1 (KpnAI); lane 3, *K. pneumoniae* GM236 (KpnBI); lane 4, *K. pneumoniae* D8; lane 5, *K. pneumoniae* OK8 (KpnI); lane 6, *E. coli* C; lane 7, *E. coli* 4001 (SB); lane 8, *E. coli* 2379 (A); lane 9, *S. typhimurium* LT2 (SA, SB, and LTI); lane 10, *E. coli* NM522 (K mutant); lane 11, *E. coli* CSH50 (K); and lane 12, 1 kb-ladder.


1
2
3
4
5
6
7
8
9
10
11
12
7.2
3.3
2.6
1.6

The hybridization of the 2.6-kb SB-specific fragment to *Klebsiella* DNA is still unexplained. It is possible that a region which codes for a common function of all enteric bacteria, such as *dam* or *dcm*, is adjacent to the *hsd* genes. This interpretation would mean that the *Klebsiella* DNA fragment selected for hybridization to the *hsd*SB gene fragment may contain DNA unrelated to restriction and modification of KpnBI.

C. Cloning by transposon (λ*plac*Mu) mutagenesis

Cloning an R-M gene by using a λ*plac*Mu transposon involves random insertion of the transposon into the host chromosome and selection for restrictionless mutants. Once the mutant is isolated, it is then possible to identify and clone an *Eco*RI fragment which contains the *lacZ* end of λ*plac*Mu and part of the inserted gene (*hsd*KpnBI). The restriction map of λ*plac*Mu at the *lacZ* terminal, which is identical to Mu dl1, is known (O'Connor and Malamy, 1983) (see Fig. 2). An *Eco*RI site is located 4.8 kb from the *lacZ* end. This *Eco*RI chromosomal-*lacZ* fusion DNA fragment can then be identified by DNA hybridization using *lacZ* as a probe. The identified DNA fragment which contains *lacZ* can be recovered and used for further cloning of the *hsd* gene.

This method is convenient and has been used to estimate the insertion locus of λ*plac*Mu in *hsd*K genes (Prakash et al., 1991). UV-induction of the integrated λ*plac*Mu lysogen also allows isolation of the genes adjacent to the transposon. Once the fragment containing both *lacZ* and part of the gene of interest is cloned and a restriction map is made, a chromosomal fragment can be isolated and used as a probe to search for an entire (functional) *hsd*KpnBI clone from a plasmid library derived from the wild-type DNA. Thus, in order to clone the *hsd*KpnBI genes by this method, construction of λp*lac*Mu fusion mutants is necessary.

From 89 r^- mutants obtained from experiments two to six (Table 8), 54 strains were selected for a DNA hybridization study with a $[^{32}P]$-*lacZ* probe. Six mutants which contained single λp*lac*Mu insertions were selected as candidates for attempts at cloning the *Klebsiella* R-M genes.

If λp*lac*Mu (which contains one *Eco*RI site) is inserted into an *hsdR* gene, different r^- mutants would be expected to yield *Eco*RI fragments of various sizes from that region. These fragments can be identified by hybridization with a *lacZ* probe. Thus, a fragment of DNA identified as part of *hsdR* by hybridization with *lacZ* could then be used as a second probe to hybridize with all the λp*lac*Mu insertions (both single and multiple) to identify the *hsdR* gene in the mutants. On the other hand, if the fragment used as a probe came from a random insertion, the chances of a shift in the migration of an *Eco*RI fragment in other λp*lac*Mu mutants should be very low.

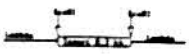
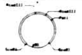
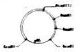
All six single λp*lac*Mu insertion mutants (4 r^-m^- and 2 r^-m^+) were used for cloning the single *Eco*RI-*lacZ*-hybridized DNA fragments into LambdaDASH™. A λ pool which contained random insertions originating from each mutant was screened for the clone containing the *lacZ* gene. Two methods of screening for *lacZ*-positive clones were performed.

In the first method, the λ clone pool (of two single r^-m^- λp*lac*Mu insertion mutants) was plated on a lawn of *E. coli* LE392 (*lacZ*$^-$) with X-Gal but without IPTG. The frequency of blue plaques obtained was about 1%. These

LambdaDASH™ clones should have contained chromosomal fragments with a *lacZ*-fusion gene. Ten representative, well-isolated blue plaques (Gal⁺) were selected and propagated again on *E. coli* LE392. Their DNA were extracted, cut with *Eco*RI, and run on electrophoresis gels. Finally the DNA was blotted onto a nitrocellulose membrane and hybridized with an *E. coli lacZ* probe. However, none of these λ clones hybridized to *E. coli lacZ*. It was therefore concluded that none of these clones contained a λ*plac*Mu-chromosomal junction fragment. Since the *lacZ* genes of *K. pneumoniae* and *E. coli* contain about 60% DNA sequence similarity (Buvinger and Riley, 1985), the clones may contain the *K. pneumoniae lacZ* gene. However, another possibility is that the clones might contain a β-galactosidase-like gene of *K. pneumoniae*, such as *ebgA* (evolved β-galactosidase) gene. Stoke et al. (1985) reported that the *ebgA* gene of *E. coli* K-12 contained 50% nucleotide similarity with the *E. coli lacZ* gene. This gene codes for an ineffective lactase, however upon the occurrence of two point mutations the enzyme is able to replace the function of the *lacZ* β-galactosidase (Hall, 1982).

An alternative method for selection of *lacZ*-positive clones is by hybridization with a *lacZ* probe. Accordingly, λ plaques from the λ clone pool which hybridized to a *lacZ* probe were selected and propagated in *E. coli* LE392. DNA was extracted from each clone and cut with *Eco*RI. Southern hybridization confirmed the presence of the *lacZ* fragment. The *Eco*RI DNA fragment was subcloned into the plasmid vector pBluescript™ and a restriction map of each clone was constructed using *Eco*RI, *Bam*HI, *Hin*dIII, and *Pst*I (Table 14).

TABLE 14. Summary of cloning of the *lacZ* junction of the six single λ*plac*Mu insertion mutants of *K. pneumoniae* GM236

Mutant #	1 (r^-m^-)	2 (r^-m^-)	3 (r^-m^+)
Clones in lambdaDash™			
Size of insert	9.5 kb	10 kb	8.2 kb
Subclones in pBluescript™			
DNA hybridization using DNA next to lacZ as a probe	No positive hybridization band was determined	No positive hybridization band was determined	All r^-m^-, r^-m^+ and wild type showed the same single band
Interpretation	part of lambda	part of lambda	other gene

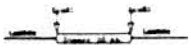
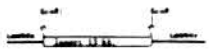
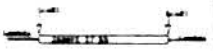
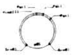
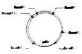
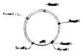
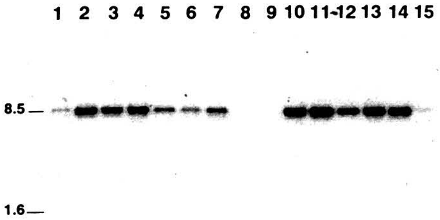
Mutant #	4 (r^-m^-)	5 (r^-m^-)	6 (r^-m^+)
Clones in lambdaDash™			
Size of insert	10 kb	13 kb	17 kb
Subclones in pBluescript™			
DNA hybridization using DNA next to lacZ as a probe	All r^-m^-, r^-m^+ and wild type showed the same single band	All r^-m^-, r^-m^+ and wild type showed the same single band	All r^-m^-, r^-m^+ and wild type showed the same single band
Interpretation	other gene	other gene	other gene

FIG. 15. Hybridization of *K. pneumoniae* GM236 chromosomal DNA with a portion of the λ*plac*Mu insertion mutant #4 DNA. Autoradiograph showing hybridization of the DNA fragment next to the *lacZ* operon fusion of a single λ*plac*Mu insertion mutant #4 (see Table 14) with the chromosomal DNA of *K. pneumoniae* GM236 λ*plac*Mu insertion mutants showing r^- phenotype (lanes 1-7 and 10-14) and the wild-type DNA of *K. pneumoniae* GM236 (lane 15). Lane 8 contains a 1-kb size marker. The DNA in lane 9 was degraded.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14
15
8.5
1.6

wild-type DNA which indicated that the probe was probably not chromosomal DNA. It is possible that the probe consisted only of Mu or λ DNA, since several hybridized bands were observed in the r^-m^- and r^-m^+ mutants as shown in Fig. 16. It was also shown that this probe hybridized to the high-molecular-weight marker which is made of λ DNA. Thus, it was concluded that the DNA fragment adjacent to *lacZ* in mutants #1 and #2 was part of λ DNA.

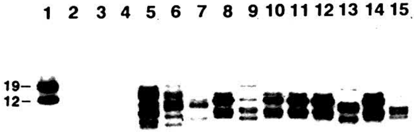
In conclusion, although a number of single λ*plac*Mu-inserted clones were isolated, none contained the KpnBI gene.

D. Cloning into plasmid pBR322

The last cloning approach was to clone *hsdR* gene into a plasmid vector. This methodology is based on the knowledge that *hsdR*-containing bacteria restrict non-modified phage, whereas bacteria without *hsdR* are lysed by non-modified phage. If the *hsdR* gene of KpnBI were to be cloned in a plasmid and transformed to an r^-m^+ recipient of the same system, the cell carrying an *hsdR* gene in the plasmid could be detected by its ability to restrict SBS.0.

This cloning approach has several advantages. First, this method allows the cloning and expression of the clone in a *K. pneumoniae* host in one step without passing through *E. coli* as is necessary with the cloning procedures used for other R-M systems (Mural et al., 1979; Fuller-Pace et al., 1984; Firman et al., 1985; Lunnen et al., 1988; and Hammond et al., 1990; De Backer and Colson, 1991). This is an important aspect to consider, as it is also possible that the *hsd* clone of *K. pneumoniae* may not be expressed in *E. coli*. Secondly, a clone which contains only the *hsdR* of type I or type III cannot be expressed unless the

FIG. 16. Hybridization of *K. pneumoniae* GM236 chromosomal DNA with a portion of the λ*plac*Mu insertion mutant #1 DNA. Autoradiograph showing hybridization of the DNA fragment next to the *lacZ* operon fusion of a single λ*plac*Mu insertion mutant #1 (see Table 14) with the chromosomal DNA of *K. pneumoniae* GM236 λ*plac*Mu insertion mutants showing r^- phenotype (lanes 5-15), GM236 wild type (lane 2), GM238 (lane 3), and GM236 carrying pTroy11 (lane 4). Lane 1 contained 1-kb DNA marker and high-molecular-weight marker (made of λ DNA).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
19–
12–

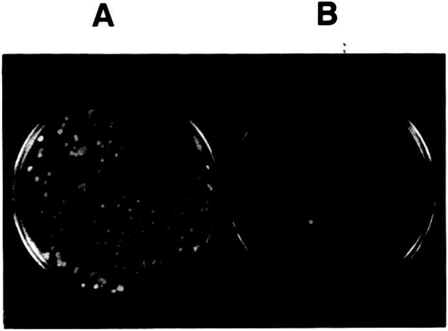
complete system is provided. This approach has to meet several conditions to be successful. An r^-m^+ strain of the system to be cloned must be isolated, and a high-efficiency method of plasmid transformation, as well as screening conditions to differentiate between the phenotypes r^+ and r^-, needs to be established.

Results of EOP in Table 6 show that SBS.0 plates at an EOP of about 10^{-3} on r^+ *K. pneumoniae* GM236 when grown at 30°C. Thus if an *hsdR*-containing plasmid in r^-m^+ GM236R expressed similar restriction activity as the wild-type GM236, a 1,000-fold enrichment might be expected. The procedure for using this cloning method was as follows.

K. pneumoniae GM236 DNA, partially digested with *Sau*3AI, was ligated into *Bam*HI-linearized, dephosphorylated pBR322 DNA. The ligated DNA was transformed into a *K. pneumoniae* GM236 r^-m^+ mutant (GM236R) by electroporation. Transformants were plated out on L agar plates containing ampicillin and incubated overnight at 30°C. The colonies were screened for the r^+_{KpnBI} phenotype by replica plating onto a lawn of SBS.0 phages. It was expected that an r^+_{KpnBI} clone would restrict the SBS.0 phage and thus survive on the plate, whereas other clones would be lysed. Any surviving transformants could then be tested for R-M phenotype with phage SBS.

Approximately 1.2×10^5 Amp^r transformants were screened. Figure 17 is a picture of a surviving colony on one of the test plates. Thirty-six Amp^r colonies which survived exposure to phage SBS.0 were tested for their R-M phenotypes. Sixteen were SBS-resistant and 18 were r^-. Two clones had the r^+_{KpnBI} phenotype. The plasmids in these clones, designated pKpnB1 and pKpnB2, were extracted

FIG. 17. Demonstration of the technique used to select for clones exhibiting the r^+m^+ phenotype. A genomic library of *K. pneumoniae* GM236 (wild type) was made using pBR322 as a vector. The plasmid pool containing random insertions was used to transform a *K. pneumoniae* GM236R mutant (r^-m^+). The transformants were plated on L agar media supplemented with ampicillin (1,000 μg/ml). The growing colonies were replica plated on L agar media supplemented with ampicillin (1,000 μg/ml) (Plate A) and L agar containing the same concentration of ampicillin but seeded with SBS.0 (Plate B). SBS.0 carries unmodified DNA for the KpnBI system which would be restricted in the presence of a functional KpnBI R-M system. Plate A shows the initial selection of *K. pneumoniae* cells which express the antibiotic resistance carried on pBR322. Plate B shows the clone which expresses both the antibiotic resistance and a functional R-M system. The background on plate B is made up of *K. pneumoniae* which has been lysed by SBS.0 and minimally growing SBS-resistant colonies.


A
B

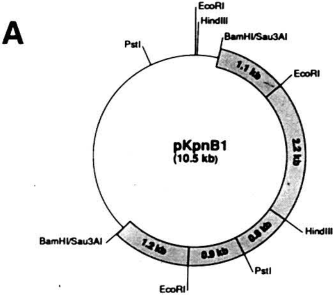
from each clone and transformed back to *K. pneumoniae* GM236R ($r^-_{\mathrm{KpnBI}}m^+_{\mathrm{KpnBI}}$) and the restriction phenotype was confirmed.

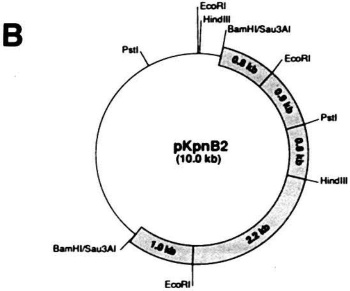
The pKpnB1 plasmid had a 6.2-kb insert and the pKpnB2 plasmid contained a shorter 5.7-kb insert. A restriction map of each plasmid was constructed for *Eco*RI, *Hin*dIII, and *Pst*I. The restriction maps of pKpnB1 and pKpnB2 are shown in Fig. 18 and demonstrate that the shorter 5.7-kb fragment of pKpnB2 is included within the larger 6.2-kb fragment of pKpnB1, but in the opposite orientation.

To determine whether the *hsdM* gene of KpnBI had also been cloned and to determine whether KpnAI and KpnBI complemented each other, the plasmids pKpnB1 and pKpnB2 were transformed into the r^-m^- strains of *K. pneumoniae* GM238 ($r^-_{\mathrm{KpnBI}}m^-_{\mathrm{KpnBI}}$ $r^0_{\mathrm{KpnAI}}m^0_{\mathrm{KpnAI}}$) and 5022 ($r^-_{\mathrm{KpnAI}}m^-_{\mathrm{KpnAI}}$ $r^0_{\mathrm{KpnBI}}m^0_{\mathrm{KpnBI}}$) and into M5a1R ($r^-_{\mathrm{KpnAI}}m^+_{\mathrm{KpnAI}}$), and the R-M phenotypes of the transformants were determined. All GM238 transformants expressed the $r^+_{\mathrm{KpnBI}}m^+_{\mathrm{KpnBI}}$ phenotype, but all 5022 and M5a1R transformants expressed only the recipient phenotypes. Thus, the plasmid-associated KpnBI genes complemented KpnBI on the chromosome but the restriction subunit of KpnBI failed to complement KpnAI. Moreover, since neither 5022 nor M5a1R were complemented by KpnBI on the plasmids, it is probable that only part of *hsdM* has been cloned. If KpnBI is a type I system, it is possible that all of *hsdM* but only part of *hsdS* have been cloned, since the product of *hsdM* and *hsdS* are both required for the functional restriction endonuclease.

Because of the possibility of similarity between KpnBI and K system, complementation between KpnBI and K was also tested. However, when the

FIG. 18. Restriction maps of clones (A) pKpnB1 and (B) pKpnB2. Inserted DNA is shown by thick shadow, while pBR322 vector DNA is shown as a single line. A pKpnB1 contains a 6.2-kb section of inserted DNA at the *Bam*HI site of pBR322, whereas pKpnB2 contains a 5.7-kb segment of inserted DNA at the same site of the vector. Restriction endonuclease sites and location are indicated.


A
EcoRI
HindIII
BamHI/Sau3AI
PstI
1.1 kb
EcoRI
pKpnB1
(10.5 kb)
2.2 kb
0.8 kb
HindIII
BamHI/Sau3AI
1.2 kb
0.9 kb
PstI
EcoRI


B
EcoRI
HindIII
BamHI/Sau3AI
PstI
0.8 kb
EcoRI
0.8 kb
PstI
pKpnB2
(10.0 kb)
0.9 kb
HindIII
BamHI/Sau3AI
1.0 kb
2.2 kb
EcoRI

pKpnB1 and pKpnB2 plasmids were transformed into *E. coli* XL1-Blue ($r^-_K m^+_K$), all transformants expressed only the $r^-_K m^+_K$ recipient R-M phenotype. Thus the restriction subunit of KpnBI failed to complement the r^- trait of the K system.

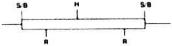
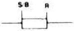
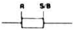
In order to determine more exactly the location of the *hsdR* in the pKpnB1 clone, different restriction fragments of pKpnB1 were subcloned into pBR322. Complementation tests of each subclone with both the $r^-_{KpnBI} m^+_{KpnBI}$ GM236R and the $r^-_{KpnBI} m^-_{KpnBI}$ GM238 were performed and the R-M phenotype of each subclone determined. The results of these complementation tests are shown in Table 15. The smallest DNA fragment to retain restriction, EOP of 10^{-3} in GM236R, was the 3.9-kb *Eco*RI fragment. However, the higher EOP (10^{-1}) and weak modification activities were obtained with GM238 recipient. It is possible that one of the *Eco*RI sites cut within *hsdM* gene.

To verify if the pKpnB1 plasmid contained any DNA sequence homologous to pBJ1, the 3.9-kb *Eco*RI fragment was used as a probe to hybridize both the 7.2-kb fragment of the pBJ1 plasmid and *Eco*RI-digested GM236 DNA. No hybridization was detected in the 7.2-kb of the pBJ1, but a single 3.9-kb band was detected in the *Eco*RI-digested GM236 DNA. This result confirms that the pBJ1 plasmid contained DNA fragments other than *hsd*KpnBI genes.

E. DNA hybridization of the 3.9-kb *Eco*RI fragment of pKpnB1 to other enteric bacterial DNA

To determine whether the KpnBI system shared any DNA homology with chromosomal DNA of other enteric bacteria, the 3.9-kb *Eco*RI fragment of pKpnB1 was labeled with [α-^{32}P]dCTP by nick translation and probed against

TABLE 15. Complementation results of pKpnB1, pKpnB2, and subclones of pKpnB1

Clones and subclones		Recipient strains				
		GM236R ($r^-_{KpnBI}m^+_{KpnBI}$)	GM238 ($r^-_{KpnBI}m^-_{KpnBI}$)	M5a1R ($r^-_{KpnAI}m^+_{KpnAI}$)	5022 ($r^-_{KpnAI}m^-_{KpnAI}$)	*E. coli* XL1 ($r^-_K m^+_K$)
pKpnB1 (6.2-kb insert)		$r^+_{KpnBI}m^+_{KpnBI}$	$r^+_{KpnBI}m^+_{KpnBI}$	$r^-_{KpnBI}m^-_{KpnBI}$ $r^-_{KpnAI}m^+_{KpnAI}$	$r^-_{KpnBI}m^-_{KpnBI}$ $r^-_{KpnAI}m^-_{KpnAI}$	$r^-_K m^+_K$
pKpnB2 (5.7-kb insert)		$r^+_{KpnBI}m^+_{KpnBI}$	$r^+_{KpnBI}m^+_{KpnBI}$	$r^-_{KpnBI}m^-_{KpnBI}$ $r^-_{KpnAI}m^+_{KpnAI}$	$r^-_{KpnBI}m^-_{KpnBI}$ $r^-_{KpnAI}m^-_{KpnAI}$	$r^-_K m^+_K$
Subclone[a] (3.9-kb insert)		$r^+_{KpnBI}m^+_{KpnBI}$	$r^+_{KpnBI}m^+_{KpnBI}$[b]	ND	ND	ND
Subclone[a] (1.2-kb insert)		$r^-_{KpnBI}m^+_{KpnBI}$	$r^-_{KpnBI}m^-_{KpnBI}$	ND	ND	ND
Subclone[a] (2.9-kb insert)		$r^-_{KpnBI}m^+_{KpnBI}$	$r^-_{KpnBI}m^-_{KpnBI}$	ND	ND	ND
Subclone[a] (1.1-kb insert)		$r^-_{KpnBI}m^+_{KpnBI}$	$r^-_{KpnBI}m^-_{KpnBI}$	ND	ND	ND

[a] These subclones were made from pKpnB1. Restriction site on the maps are S/B (*Sau*3AI/*Bam*HI), H (*Hin*dIII), and R (*Eco*RI).
[b] The restriction and modification activity are weak (EOP about 0.1 to 0.2).
ND, not done.

*Eco*RI-digested chromosomal DNA of *K. pneumoniae* M5a1 (KpnAI), *K. pneumoniae* 5022 (KpnAI), *E. coli* CSH50 (K), *E. coli* 2379 (A), *E. coli* 4001 (SB), *S. typhimurium* LT2 (SA, SB, and LTI), *E. coli* B (B), and *E. coli* C. *K. pneumoniae* GM236 (KpnBI) and GM238 (KpnBI) were used as positive controls. The results of these hybridizations are shown in Fig. 19. Only *K. pneumoniae* GM236 and *K. pneumoniae* GM238 contained a DNA fragment (3.9 kb) which hybridized with the probe and no other strains showed any DNA homology to the 3.9-kb probe.

The same probe was also used to hybridize with other clones of *hsd* genes in the plasmid vectors P1, P15, Eco124/3, and LTI. None of the *hsd* clones showed DNA homology with the 3.9-kb probe. The hybridization bands in lanes 1, 12, 13, and 15 were due to plasmid vector DNA contamination in the 3.9-kb *Eco*RI probe.

These hybridization results further confirmed that the KpnBI system is unique and shares no homology with KpnAI, other enteric bacterial DNA, or other R-M systems tested.

To assist in the cloning of a gene(s) encoding a functional modification activity, further hybridizations between a 3.8-kb *Hin*dIII fragment from pKpnB1 (see Fig. 18) and wild-type GM236 DNA cut with different type II restriction enzymes were performed. The results of these hybridization are shown in Fig. 20. Several restriction enzymes cut the GM236 DNA and showed a single positive hybridization band, these include *Bam*HI (7.0 kb), *Bgl*II (about 17 kb), *Hin*dIII (4.9 kb), and *Pst*I (7.3 kb). Other restriction enzymes, *Eco*RI, *Pvu*II, and *Kpn*I showed 2, 2, and 3 positive hybridization bands, respectively. From these

FIG. 19. Hybridization of the 3.9-kb fragment with chromosomal DNA and plasmid clones of different R-M systems. Autoradiograph showing hybridization of the $[^{32}P]$-3.9 kb *Eco*RI fragment of pKpnB1 with *Eco*RI-cut genomic DNA of different enteric bacteria and different plasmid clones of type I and type III R-M systems. Numbers on the top indicate lanes, while numbers on the side represent DNA size markers in kb. Lanes 1 and 15 are 1-kb DNA markers; lane 2 is *K. pneumoniae* GM236 (KpnBI); lane 3 is *K. pneumoniae* GM238 (KpnBI); lane 4 is *K. pneumoniae* M5a1 (KpnAI); lane 5 is *K. pneumoniae* 5022 (KpnAI); lane 6 is *E. coli* CSH50 (K); lane 7 is *E. coli* 2379 (A); lane 8 is *E. coli* 4001 (SB); lane 9 is *S. typhimurium* LT2 (SA, SB, and LTI); lane 10 is pRH212 (P1) cut with *Bam*HI; lane 11 is pSHI1180 (P15) cut with *Bam*HI; lane 12 is p521 (LTI) cut with *Pst*I and *Sal*I; lane 13 is p531 (LTI) cut with *Sal*I.

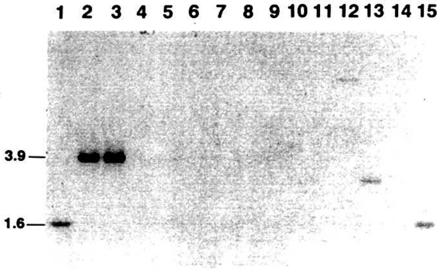
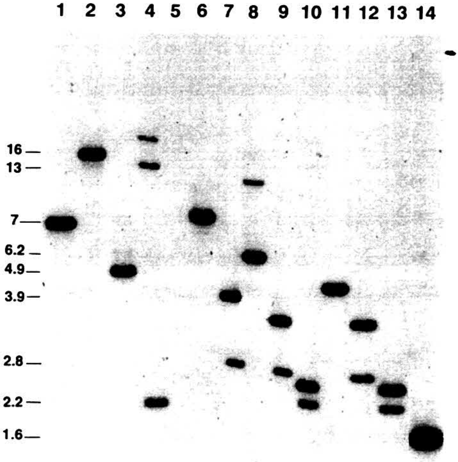

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
3.9
1.6

FIG. 20. Autoradiograph of *K. pneumoniae* GM236 (lanes 1-4 and 6-11) and GM238 (lanes 12 and 13) genomic DNA cut with different restriction endonucleases and hybridized with a $[^{32}P]$-3.8 kb *Hin*dIII fragment of pKpnB1. The numbers on the top indicate lanes, while the numbers in the side represent the DNA size markers in kb. Lane 1, *Bam*HI; lane 2, *Bgl*II; lane 3, *Hin*dIII; lane 4, *Kpn*I; lane 5, λ-*Hin*dIII DNA size markers; lane 6, *Pst*I; lane 7, *Eco*RI; lane 8, *Pvu*II; lane 9, *Eco*RI and *Bam*HI; lane 10, *Eco*RI and *Hin*dIII; lane 11, *Hin*dIII and *Pst*I; lane 12, *Eco*RI and *Bam*HI; and lane 13, *Eco*RI and *Hin*dIII.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
16
13
7
6.2
4.9
3.9
2.8
2.2
1.6

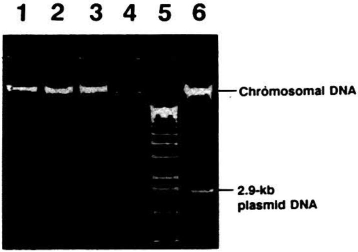
hybridization results it could be concluded that *Eco*RI, *Pvu*II, and *Kpn*I probably have a restriction site within *hsdM*, but that the *Bam*HI, *Bgl*II, *Hin*dIII, and *Pst*I sites are beyond *hsdM*. This information could be useful for the further cloning of the KpnBI modification gene(s) or the complete *hsd*KpnBI genes, and the physical mapping of this region.

Is KpnBI on the chromosome or on a plasmid?

Many plasmids are carriers of type II R-M systems as shown with *Eco*RI (Greene et al., 1981; Newman et al., 1981), *Eco*RII (Kosykh et al., 1980), *Eco*RV (Bougueleret et al., 1984), *Eco*VIII (Mise and Nakajima, 1984), *Pvu*II (Blumenthal et al., 1985), *Sbo*13 and *Eco*T22 (Mise et al., 1986), and *Pae*R7 (Theriault and Roy, 1982; Gingeras and Brooks, 1983). Electrophoresis of plasmid DNA extracts from M5a1 and GM236 did not reveal a plasmid (Fig. 21). Although the presence of a large plasmid in the strains was not excluded, these results along with results presented earlier (in Results Part II, section D and E) suggest that it is highly improbable that the *hsd* genes coding for these two R-M systems are located on a plasmid.

To obtain evidence that KpnBI was chromosomally located, the 3.9-kb *Eco*RI fragment of *hsd*KpnBI was hybridized to *Xba*I-digested GM236 chromosomal DNA (derived from experiments being conducted by A. Randriamahefa). It hybridized with a 40-kb chromosomal fragment. This result would appear to confirm the chromosomal location of KpnBI.

FIG. 21. Agarose gel electrophoresis of plasmid extracts from *K pneumoniae* strains M5a1, GM236, D8, and OK8. *E. coli* JM109 containing pGem-3Z (lane 6) were used as a positive control. Lane 1, M5a1; lane 2, GM236; lane 3, D8; lane 4, OK8; and lane 5, 1-kb DNA marker.


1
2
3
4
5
6
Chromosomal DNA
2.9-kb plasmid DNA

DISCUSSION

The major result of this research project is the cloning of the ***hsdR*** gene of the KpnBI restriction-modification system of *Klebsiella pneumoniae* GM236. The successful cloning of the *K. pneumoniae* ***hsdR*** gene was accomplished using a cloning procedure in which DNA fragments from the chromosome of the source strain GM236 were ligated into the plasmid vector pBR322. The ligation mixture was electroporated into an r^-m^+ *K. pneumoniae* host (GM236R) and transformants were screened to detect expression of KpnBI restriction activity (see Results Part II, section D, for details).

This procedure has several advantages over previously used methods. One of these advantages is the ability to clone and express the gene of interest in one step without passing it through *E. coli*. This eliminates the concern that the ***hsdR*** gene of *K. pneumoniae* may not be expressed in *E. coli*. In addition, this method detects only those clones that contain the complete, fully-expressible target gene. Several tools needed to be constructed before this approach could be used. An r^-m^+ strain of the KpnBI system was developed for use as a recipient of the ***hsdR*** gene. This r^-m^+ strain allowed direct screening for expression of the ***hsdR*** gene. Secondly, a reliable and reproducible screening method for discrimination between the r^+ and r^- phenotypes was established. Finally, a highly efficient plasmid transformation method for *K. pneumoniae* was also employed.

Three transformation methods, $CaCl_2$ heat-shock, freeze-thaw cycle, and electroporation, were evaluated in this study. The electroporation method of plasmid transformation was found to be the most efficient for *K. pneumoniae* strains. The efficiency of electroporation into *K. pneumoniae* varied from 10^4 to 10^6 CFU/μg DNA (Table 10). It is likely that this variability was a consequence of different plasmid sizes and DNA purity because the same method produced 10^7 to 10^8 transformants per μg of DNA when commercially prepared pBR322 DNA was used. However, this level of transformation is still relatively low compared to the 10^9 to 10^{10} transformants/μg DNA obtained with *E. coli* (Dower et al., 1988). It may be possible to improve the efficiency of electroporation into *K. pneumoniae* by determining the optimal conditions for the procedure. Variables such as electrical field strength, pulse length, cell concentration, and DNA concentration could be examined. Only the set of conditions recommended by the manufacturer for electroporation into *E. coli* was used in this study. Other conditions which may improve *K. pneumoniae* transformation include using a *galE* recipient strain or preparing competent cells from different stages of growth. These two parameters have been examined in other bacteria and led to significant improvements in transformation efficiency (Brown et al., 1979; Hanahan, 1983; MacLachlan and Sanderson, 1985; Miller et al., 1988; Tsai et al., 1989; Inoue et al., 1990). Another aspect of the electroporation method that I have observed to be important in this study is that the transformation efficiency is greatly reduced by salts and other chemicals generally found in ligation reaction mixtures. The removal of salt and other chemicals prior to electroporation is essential for optimal efficiency and to minimize the occurrence of electrical arcing.

Interestingly, the observed occurrence of *hsdR* clones was lower than expected. Assuming that the genome size of *K. pneumoniae* is approximately 5,000 kb (A. Randriamahefa, personal communication) and that the average size of cloned fragments in the plasmid vector was about 5 kb, we would expect one successful *hsdR* clone in every 1,000 transformants screened (0.1%). However, the frequency of *hsdR*$^{+}$ clones observed in this study was 0.002%. There are several possible explanations for the low frequency of clone occurrence: (i) the *Sau*3AI library may not be completely random; some fragments may not be adequately represented; (ii) some *Sau*3AI fragments may contain genes for products which are lethal when cloned into multicopy plasmids; (iii) *Sau*3AI fragments containing the *hsdR* gene may also include a sequence which blocks plasmid replication; (iv) multiple copies of plasmid containing the restriction gene may be harmful to the recipient cells; and (v) there may have been a technical flaw in the screening process in that there were approximately 1,000 transformants per plate used in replica plating. During the screening of these plates non-modified SBS phages were used to enrich for r^{+}_{KpnBI} clones. These phages were expected to infect and kill $r^{-}_{KpnBI}m^{+}_{KpnBI}$ clones, but not $r^{+}_{KpnBI}m^{+}_{KpnBI}$ clones. However, if the clones were too close together the phages released by lysis of $r^{-}_{KpnBI}m^{+}_{KpnBI}$ clones would have the KpnBI modification and would be able to kill the r^{+}_{KpnBI} clones as well. A similar observation has been reported by Rodicio and Chater (1988) in cloning the *Sal*I restriction and modification genes of *Streptomyces albus* G. The chance of identifying *hsdR* clones of *K. pneumoniae* would have been increased if the number of colonies had been reduced (to about 300 per plate) on the plates used for replica plating and screening. Another possible reason for obtaining fewer transformants than expected may be due to the restriction enzyme (*Sau*3AI) used to digest the bacterial genomic DNA. If the enzyme cuts many times within the *hsdR* gene, intact clones would be rare, even though a procedure for partial digestion was used (Lunnen et al., 1988). To circumvent this possible difficulty one can make libraries using one of several different restriction enzymes. Although this strategy would increase the workload, it may substantially increase the chance of success.

Several different cloning strategies were used in attempts to clone the *hsd* genes of *K. pneumoniae*. These methods did not yield any *hsd* clones under the conditions described in this study. These procedures included the use of λ as a cloning vector. In that case, it was found that λ failed to propagate in *K. pneumoniae* containing pTroy11 (pTroy11 carries the λ receptor gene *lamB*). A similar difficulty was reported in *S. typhimurium* (Schwartz and Le Minor, 1975). In *Salmonella*, several conditions, other than the presence of λ receptors, are required for λ to propagate (C. Colson, personal communication; De Backer and Colson, 1991). These factors include a mutation of the *galE* (galactose epimerase) gene (De Backer and Colson, 1991) and an intact *nusA* gene which produces an anti-terminator required for the function of the λ N-protein leading λ to a lytic cycle (Harkki and Palva, 1984). Further study is needed to reveal all of the conditions needed to propagate λ in *K. pneumoniae* strains.

Another cloning attempt was based upon genetic homology. I endeavored to detect the *hsd* genes of *K. pneumoniae* by Southern hybridization with *hsd* gene probes from different sources. A single 7.2-kb *Eco*RI-digested chromosomal DNA fragment from *K. pneumoniae* GM236, which hybridized with *hsd* probes of the SB and K systems, was selected as a probable carrier of the *hsd* genes. However, when the 7.2-kb DNA fragment was cloned into a plasmid vector, and named pBJ1, this fragment did not express any restriction or modification activity. Furthermore, when subsequently checked against the successful *hsdR* clone obtained by a different strategy, no sequence similarity was detected by Southern hybridization. This fragment probably came from a site on the chromosome different from the location of the *hsd* genes, and may include a sequence that shows some unexplained sequence similarity to the *hsd*SB probe that was used.

A third cloning method employed mutagenesis using λp*lac*Mu. This transposon was inserted at random into the *K. pneumoniae* chromosome and the mutants were tested for restriction and modification phenotypes. The results suggest that this approach may have been complicated due to rearrangements of the λp*lac*Mu subsequent to its insertion into the *K. pneumoniae* chromosome. This phenomenon has been observed in other bacteria. For example, Barr et al. (1990) reported that when λp*lac*Mu was inserted into the chromosome of *E. coli*, it can cause chromosomal inversions at a high frequency due to the presence of a portion of the *trp* operon in λp*lac*Mu.

Although several of the cloning attempts were unsuccessful, a useful, quick subcloning method was developed. Subcloning is usually performed by isolating a DNA fragment and ligating it with a linearized plasmid vector (Maniatis et al., 1982; Davis et al., 1986; Ausubel et al., 1987). In the quick method devised in this work, the DNA fragment need not be isolated from the restriction digest of the original clone nor from the vector in which it resided. The method saves both time and reagents. In addition, by minimizing the amount of handling, the

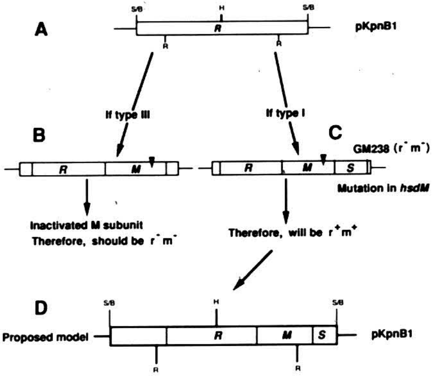
DNA losses that are common in other procedures are greatly reduced. This procedure, however, requires that the original clone be made in a plasmid vector such as pBluescript™ or pUC derivatives which contain multiple cloning sites. Two suitable restriction enzymes which will cut in the polylinker region at the right and the left of the cloned site should be selected to make the subclones. This requirement may not be a severe limitation, since many of commonly used plasmid vectors contain multiple cloning sites. After a clone that contains the desired DNA fragment has been constructed using this method, it is advisable to confirm the identity of the cloned fragment by an independent method.

An interesting observation made in the λ*plac*Mu mutagenesis studies was that approximately equal numbers of r^-m^- and r^-m^+ mutants were obtained after random selection and testing (see Results Part I, section D). Similar results were obtained previously when nitrosoguanidine was used as a mutagen (Valinluck et al., 1989). In a different study, Prakash et al. (1991) constructed *hsd*-Mu(*lac*) operon fusion mutants of *E. coli* K-12 (type I R-M system). In that study, of 110 r^-_K mutants, 39 were $r^-_K m^+_K$ and 71 were $r^-_K m^-_K$. These numbers of mutants are similar to the numbers of mutants obtained from the mutagenesis of the KpnAI and the KpnBI systems of *K. pneumoniae*. The similarity between the results in these two different studies suggests that both the KpnAI and KpnBI are type I (which consists of three genes [*hsdR*, *hsdM*, and *hsdS*]) or type III (which contains only two genes [*hsdR* and *hsdM*]), but not type II R-M systems. An r^-m^- mutant can be obtained in one step in the case of type I, and the mutation may occur at either the *hsdM* or the *hsdS* gene, whereas in type III, the mutation must be at the *hsdM*. In type II, the restriction endonuclease and modification methylase function independently, and the occurrence of an $r^{-}m^{-}$ mutant requires two independent mutations. Consequently, $r^{-}m^{-}$ mutants occur very rarely.

In this study, the *hsdR* gene of the KpnBI system was cloned into a pBR322 vector. Two independently derived clones were named pKpnB1 (6.2-kb insert) and pKpnB2 (5.7-kb insert). Both clones complement the restriction activity of the $r^{-}_{KpnBI}m^{+}_{KpnBI}$ mutant (GM236R). In addition, the two clones can complement both the restriction and modification defects of the $r^{-}_{KpnBI}m^{-}_{KpnBI}$ mutant (GM238). This indicates that not only *hsdR*, but either *hsdM* or *hsdS* (but not both), is also present in the clones, and that the GM238 mutant probably has a single genetic defect located either in the *hsdM* or the *hsdS* genes (assuming that it is a type I system; Fig. 22). Since neither of the two clones expressed KpnBI restriction or modification in *K. pneumoniae* 5022 ($r^{-}_{KpnAI}m^{-}_{KpnAI}$), it is clear that the clones do not contain the entire *hsd*KpnBI system.

The complementation data are most consistent with KpnBI being a type I R-M system. Type III systems contain only two genes coding for R and M subunits. Modification can be performed independently by the M subunit alone or by the complex of M and R subunits. Restriction activity, however, is limited exclusively to the complex; both subunits must be active in order for restriction to occur. In such a situation, GM238 with $r^{-}m^{-}$ phenotype is presumed to have a single defect in the M subunit (a single defect in the R subunit of GM238 is discounted because such a mutant would exhibit an $r^{-}m^{+}$ phenotype.). Following this logic, the successful clones carrying the KpnBI *hsdR* gene would not complement GM238 at all. Both the clone and GM238 have intact *hsdR* genes, but neither of them produce active M product. The combination would continue

FIG. 22. A proposed model of the KpnBI system and complementation between pKpnB1 and *K. pneumoniae* GM238 ($r^{-}_{KpnBI}m^{-}_{KpnBI}$). (A): The pKpnB1 plasmid clone expressing a functional *hsdR* product, *R* in the box showing a location of *hsdR*. (B): A box containing *R* and *M* represents genes on the chromosome coding for *hsd* genes (*hsdR* and *hsdM*)in a type III system. (C): A box containing *R*, *M*, and *S* represent the region on chromosome coding for *hsd* genes (*hsdR*, *hsdM*, and *hsdS*) in a type I system. (D): A proposed model for the KpnBI system which most likely contains 3 genes shown in the box. The plasmid pKpnB1 more likely contains an incomplete *hsdS* gene. A dark triangle sign showing a mutation site in the GM238 mutant (B and C), whereas S/B, H, and R outside the box are *Sau*3AI/*Bam*HI, *Hind*III, and *Eco*RI restriction sites (A and D), respectively.


A
S/B
H
S/B
R
pKpnB1
R
R
If type III
If type I
B
C
GM238 ($r^- m^-$)
R
M
R
M
S
Mutation in *hsdM*
Inactivated M subunit
Therefore, should be $r^- m^-$
Therefore, will be $r^+ m^+$
D
S/B
H
S/B
Proposed model
R
M
S
pKpnB1
R
R

to be r^-m^-. Since the experiment showed that complementation does occur, the type III model must be invalid. Conversely, the complementation data conform to a three-gene model (type I system). Complementation with GM236R (r^-m^+) indicates that the clones carry intact *hsdR* genes. They are also presumed to carry the complete *hsdM* gene because they complement GM238 (r^-m^-). However, they lack all or part of the *hsdS* gene because they do not express any function on their own.

Complementation studies of the 3.9-kb *Eco*RI and 1.1-kb *Eco*RI-*Bam*HI/*Sau*3AI subclones of pKpnB1 (Table 15) showed that the 3.9-kb *Eco*RI subclone still contains an intact *hsdR* gene which can complement the mutation in the r^-m^+ GM236R strain, but restriction and modification activity in r^-m^- GM238 mutant was weak (EOP of 0.1 to 0.2). Meanwhile, a subclone of the 1.1-kb *Eco*RI-*Bam*HI/*Sau*3AI fragment showed neither restriction nor modification in the r^-m^+ GM236R mutant or in the r^-m^- GM238 strain. It is possible that *Eco*RI cut inside the *hsdM* gene in the pKpnB1 clone. Thus, the 3.9-kb *Eco*RI subclone should contain a portion of the *hsdM* gene and the 1.1-kb *Eco*RI-*Bam*HI/*Sau*3AI subclone should contain the other portion of the *hsdM* and probably part of the *hsdS* as shown in Fig. 22. According to this model the defect in strain GM238 should be within the *hsdM* gene.

Further complementation studies also showed that KpnBI is different from KpnAI, since pKpnB1 and pKpnB2 did not complement either the restriction or modification phenotype of the $r^-_{KpnAI}m^-_{KpnAI}$ strain, 5022, or the $r^-_{KpnAI}m^+_{KpnAI}$ strain, M5a1R (Table 15). In addition, no DNA homology between the M5a1 chromosomal DNA and the 3.9-kb *Eco*RI fragment of pKpnB1 probe was detected (Fig. 19). Similar results were obtained when complementation between KpnBI system and K system of *E. coli* was studied (Table 15). DNA hybridization studies between the cloned KpnBI and K, A, SB, SA, LTI, P1, P15, and Eco124/3 showed no DNA homology (Fig. 19). All these results indicate that the KpnBI system is unique.

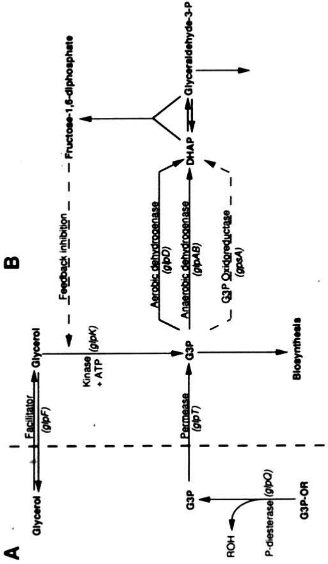
This work also revealed the effects of temperature and glycerol concentration on KpnBI restriction activity. While little is known about the physiological conditions that promote the loss of the ability to restrict, it was shown in this study that the restriction activity of the KpnBI system was decreased when the cells were cultured at a higher temperature (42°C) or in the presence of 10% glycerol.

It has been shown clearly (Table 6 and Fig. 6) that the *Kpn*BI restriction endonuclease, but not the modification methylase, is heat labile, and that the temperature sensitivity is a characteristic of the enzyme's catalytic activity and not a consequence of altered gene expression. Uetake et al. (1964) have shown that when *S. typhimurium* is exposed to temperatures of 45 to 50°C for short periods of time, there is a breakdown in the mechanism of restriction. This effect lasts for one generation while the bacteria recover the ability to restrict bacteriophage. In *S. typhimurium*, two R-M systems have been reported as thermolabile (Colson and Van Pel, 1974). The SB system is temperature-sensitive with respect to both restriction and modification, whereas the SA system is temperature-sensitive with respect to restriction only. A similar temperature effect on the R-M system in *Pseudomonas aeruginosa* was reported by Holloway (1965). *P. aeruginosa* grown at 43°C lost its ability to restrict. Remarkably, the ability to restrict was not restored for 60 to 70 generations of growth at 37°C.

The effect of glycerol on *Kpn*BI restriction activity is a new and surprising observation. The mechanism of action of glycerol on the restriction endonuclease is still unknown. It may be a direct effect of glycerol itself or a consequence of glycerol metabolism. Glycerol may cause a change in osmotic pressure or viscosity which may affect the folding or tertiary structure of proteins. Seigneuret et al. (1991) reported that glycerol had a large effect on the motion of the outer peptide side chain of the proton transport membrane protein (bacteriorhodopsin).

Assuming the glycerol transport mechanisms of *Klebsiella* and *E. coli* are similar, glycerol can enter the cytoplasm by facilitated diffusion across the cytoplasmic membrane. The facilitator protein (encoded by the *glp* regulon) provides a selective channel with an estimated pore size of 0.4 nm (Eze and McElhaney, 1981) (Fig. 23). Once inside the cell, glycerol can be trapped as glycerol-3-phosphate by the action of the same ATP-dependent kinase that also phosphorylates dihydroxyacetone (Hayashi and Lin, 1967). Inside the cell, glycerol or its metabolites may affect cell activities. Genetic mutants of the *glp* regulon can be used to determine whether glycerol itself or one of its metabolites affects restriction activity. The requirement for glycerol entry into the cell may be tested by using a *glpF-glpT* mutant (Fig. 23), while a *glpK-glpT* mutant could be used to differentiate between the effect of glycerol or its metabolites. Shimizu and Katsura (1988) reported that 15% glycerol can reduce by 50% the activity of the adenosine triphosphatase (ATPase) of dynein extracted from *Tetrahymena* cilia. The effect was attributed to glycerol itself. Glycerol also exhibited

FIG. 23. Metabolic pathways of glycerol and glycerol-3-phosphate (G3P). A discontinued vertical line represents the cytoplasmic membrane, whereas "A" and "B" indicate the outside and inside of bacterial cell respectively. Glycerophosphodiesters (G3P-OR) are hydrolysed to G3P by a phosphodiesterase, and G3P is actively transported into the cytoplasm by a permease activity. (From Lin, 1987)


A
Glycerol
Facilitator
(glpF)
Glycerol
Kinase
+ ATP
(glpK)
G3P
Permease
(glpT)
G3P
P-diesterase
(glpQ)
G3P-OR
ROH
Biosynthesis
B
Feedback inhibition
Aerobic dehydrogenase
(glpD)
Anaerobic dehydrogenase
(glpAB)
G3P Oxidoreductase
(gpsA)
DHAP
Fructose-1,6-diphosphate
Glyceraldehyde-3-P

a decelerating effect on the rate of oxygen exchange between phosphate and water catalyzed by dynein in the presence of ADP and Mg^{2+} (Shimiza and Katsura, 1988). Glycerol induces spore formation in *Myxococcus xanthus* (O'Connor and Zusman, 1991). However, in the present study, the glycerol effect was observed only on the restriction endonuclease activity of KpnBI, but not in the KpnAI system. Further examination of the effects of glycerol on R-M systems may be fruitful. Other compounds that change osmotic pressure might be used to determine if the effect of glycerol is a function of the compound itself or an indirect effect of change in osmotic pressure.

Several other areas of study could be developed from the observations reported here. The cloned *hsdR* gene of KpnBI system make it possible to perform the crucial experiments needed to answer the questions posed earlier. Clones expressing m^+ or r^+m^+ activity can be enriched from the *K. pneumoniae* GM236 ($r^+_{KpnBI}m^+_{KpnBI}$) chromosomal DNA plasmid (pBR322) library in the recipient *K. pneumoniae* 5022 ($r_{KpnAI}m_{KpnAI}$) using the 3.8-kb *Hin*dIII fragment of pKpnB1 as a probe. *K. pneumoniae* 5022 transformants that hybridize can be tested for KpnBI restriction and modification activity. These clones will be useful for further DNA and protein studies described below.

The *hsd*KpnBI genes can be localized on the cloned DNA fragment by constructing double-strand, nested deletions of a pKpnB1 (r^+ clone), and m^+ clone and r^+m^+ clones once they are isolated. A commercially available ExoIII/mungbean nuclease kit (Stratagene) may be used for this purpose,.

The r^+, m^+, or r^+m^+ clones can then be sequenced by the Sanger dideoxy termination method using Sequenase™ (United States Biochemical Co.). Once the DNA sequences have been determined, potential open reading frames, ribosome binding sites, promotor regions, and stop codon regions can be identified. Furthermore, the *hsd*KpnBI DNA and amino acid sequences can be compared with other known *hsd* sequences using a computer database (GenBank and EMBL).

A study of the polypeptides encoded by the *hsd*KpnBI genes and the determination of the DNA recognition sequences of the KpnBI enzymes will provide useful information for the classification of the KpnBI R-M system. Several experiments can be done for this purpose. Proteins encoded by the *hsd*KpnBI genes from the clone containing and expressing modification activity and from the clone containing the entire *hsd*KpnBI genes could be isolated. The purified enzyme from the entire *hsd*KpnBI genes can then be used to identify the DNA recognition sequence using the procedure of Nagaraja et al. (1985c). Additionally, ATPase assays using the methods described by Suri and Bickle (1985) and by Hadi et al. (1983) could be used. The purified methylase enzyme may be used in a modification methylase assay, following the method of Piekarowicz et al. (1985).

Once the *Kpn*BI restriction endonuclease has been identified *in vitro*, it may be easier to study the effect of glycerol on restriction activity. Furthermore, a study of the regulation of the *hsd* genes and a study of the interaction between DNA and the polypeptides encoded by the *hsd* genes, including the assembly of the protein subunits, can be performed.

The same strategy for cloning and characterization may be applied to other restriction-modification systems.

In summary, this research project described the cloning and partial characterization of a restriction-modification system in *K. pneumoniae* GM236 and M5a1. The *hsdR* gene of the KpnBI system of *K. pneumoniae* GM236 was successfully cloned and expressed. The uniqueness of the KpnBI system was established by complementation and Southern hybridization studies. Essential transformation and mutagenesis tools used in the genetic manipulation of these *K. pneumoniae* strains were also developed. The results and observations made during these studies were used to construct a likely model of the gene organization in the KpnBI R-M system.

APPENDIX

Buffers:

B Buffer (Silhavy et al., 1984)

KH_2PO_4	3.0 g
NaH_2PO_4	7.0 g
NaCl	4.0 g
$MgSO_4$	0.2 g

Add distilled water to 1 liter.

Lambda dilution buffer (Maniatis et al., 1982)

Tris-base (tris [hydroxymethyl] aminomethane)	1.21 g
$MgSO_4.7H_2O$	1.2 g
NaCl	2.92 g
Gelatin	0.1 g

Dissolve in 800 ml distilled water, then adjust pH to 7.4 with concentrated HCl. Add distilled water to 1 liter.

5x Stopping buffer (Maniatis et al., 1982)

0.25% (wt/vol) Bromophenol blue
25% (vol/vol) Glycerol
100 mM EDTA

6x Loading buffer (Maniatis et al., 1982)

0.25% (wt/vol) Bromophenol blue
30% (wt/vol) Glycerol

10x Ligase buffer (Maniatis et al., 1982)

0.66 M Tris-HCl (pH 7.5)
50 mM $MgCl_2$
50 mM Dithiothreitol
10 mM ATP

10x Dephosphatase buffer (Maniatis et al., 1982)

0.5 M Tris-HCl (pH 9.0)
10 mM $MgCl_2$
1 mM $ZnCl_2$
10 mM Spermidine

10x Restriction endonuclease buffers

Reaction buffers were provided by the manufacturers as 10x stock solutions.

Denaturization buffer (Maniatis et al., 1982)

0.5 M NaOH
1.5 M NaCl

Neutralizing buffer (Maniatis et al., 1982)

1.0 M Tris-HCl (pH 8.0)
1.5 M NaCl

10x Low stringency washing buffer (Maniatis et al., 1982)

NaCl	175.2 g
Sodium citrate	88.2 g
Sodium dodecyl sulfate	5.0 g
Sodium pyrophosphate	1.0 g

Add distilled water to 1 liter.

20x High stringency washing buffer (Maniatis et al., 1982)

NaCl	175.2 g
Sodium citrate	17.6 g
Sodium dodecyl sulfate	10.0 g
Sodium pyrophosphate	2.0 g

Add distilled water to 1 liter.

SM buffer (Maniatis et al., 1982)

NaCl	5.8 g
$MgSO_4.7H_2O$	2.0 g
1.0 M Tris-HCl, pH 7.5	50 ml
2% Gelatin	5 ml

Add distilled water to 1 liter.

TE (Tris-EDTA) buffer (Davis et al., 1986)

10 mM Tris-HCl (pH 7.4)
0.1 mM EDTA

DNA extraction buffer (Silhavy et al., 1984)

50 mM Tris-HCl (pH 8.0)
50 mM EDTA

Other solutions:

2.5x Prehybridization solution

0.25 M PIPES (piperazine-N,N'-bis [2-ethanesulfonic acid], pH 7.0)
2.0 M NaCl
0.25% SDS
0.25% Ficoll
0.25% Polyvinyl pyrolidone (PVP)
0.25% Bovine serum albumin
500 μg/ml Sheared salmon sperm DNA

A 1x prehybridization mixture is made by mixing 2 volumes of 2.5x prehybridization mixture with 2 volumes of formamide and 1 volume of distilled water.

Hybridization solution

Same as 1x prehybridization mixture.

40x TAE (Tris-acetate EDTA) (Davis et al., 1986)

Tris base	193.6 g
Sodium acetate, anhydrous	65.6 g
Sodium EDTA	13.5 g

Add 800 ml of distilled water and adjust pH to 7.2 with approximately 50 to 55 ml concentrated HCl, then add distilled water to 1 liter.

20x SSC (Davis et al., 1986)

NaCl	175.2 g
Sodium citrate	88.2 g

Add 800 ml of distilled water and adjust pH to 7.0 with HCl, then add distilled water to 1 liter.

10% Glycerol

Dissolve approximately 10 ml of concentrated glycerol in 90 ml of distilled water.

10% X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) solution

Dissolve 100 mg of X-Gal in 1 ml of dimethylformamide (DMF)

100 mM IPTG (isopropyl-β-D-thiogalactopyranoside)

Dissolve 23.8 mg of IPTG in 1 ml of distilled water

Media:

L broth (Silhavy et al., 1984)

Bacto tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Dextrose	1.0 g

Add distilled water to 1 liter and sterilize by autoclaving at 120°C and 15 psi for 30 min.

L agar

Same as L broth with the addition of 15 g of bacto agar.

Maltose broth

Same as L broth, but with 0.4 % maltose substituted for dextrose.

Soft agar

0.75% (wt/vol) of Bacto agar in distilled water.

SOC medium (Hanahan, 1983)

2% Bacto tryptone
0.5% Bacto yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM Glucose

Antibiotic containing media

An antibiotic stock solution was filter-sterilized using a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and was added at the desired concentration to a cooled 1.5% L-agar, while still in a liquid atate.

Sephadex G50 (Maniatis et al., 1982)

Add 30 g of Sephadex G50 to 250 ml of TE buffer (pH 8.0) in a 500-ml container. Make sure the powder is well dispersed. Let stand overnight at room temperature. Decant the supernatant and replace with an equal volume of TE buffer (pH 8.0).

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ABSTRACT

CHARACTERIZATION OF RESTRICTION-MODIFICATION SYSTEMS IN *Klebsiella pneumoniae*

by

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Two restriction-modification (R-M) systems, KpnAI and KpnBI, found in *Klebsiella pneumoniae* strains M5a1 and GM236, respectively, have been studied and confirmed to be different from other R-M systems reported in *K. pneumoniae*. Mutant studies suggest that the KpnAI and KpnBI systems may belong to either a type I or type III system, since approximately equal numbers of r^-m^+ and r^-m^- mutants were obtained. However, a DNA hybridization study using representative type I and type III probes from *E. coli* and *S. typhimurium* failed to show homologies to either KpnAI or KpnBI. The restriction endonuclease *Kpn*BI was found to be temperature-sensitive with maximum restriction activity at 30°C and no restriction activity at 42°C. Further, the activity of endonuclease *Kpn*BI was found to be reduced to almost zero level by growing the bacteria in the presence of 10% glycerol. Although the mechanism is not known, this is the first time such a phenomenon has been observed in any of the reported R-M systems. These studies also compared the efficiency of transformation in *K. pneumoniae* of three plasmid transformation methods; $CaCl_2$ heat-shock; freezing and thawing in the presence of $CaCl_2$; and electroporation. Electroporation was shown to be the most efficient method. Transformation efficiency in both the r^+_{KpnAI} and r^+_{KpnBI}

strains was 20- to 100-fold less than the transformation efficiency of the r^- strains, depending on plasmid size. Four different approaches have been used to clone the *hsd* genes of the KpnBI system. Two clones were obtained; these were named pKpnB1 and pKpnB2. The pKpnB1 and pKpnB2 clones were found to complement the restriction activity of a $r^-_{KpnBI}m^+_{KpnBI}$ *K. pneumoniae* mutant and were also found to complement both the restriction and modification activities of a $r^-_{KpnBI}m^-_{KpnBI}$ *K. pneumoniae* mutant. A quick subcloning method which involves making subclones from a plasmid clone in a single step was also developed.
A preliminary analysis, based on complementation studies, of the gene structure suggested that the KpnBI system may consist of three structural genes, a characteristic of the type I R-M system.