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A Physical Map of the KLEBSIELLA PNEUMONIAE M5a1 Genome

Alexandrine Randriamahefa

ABSTRACT

A PHYSICAL MAP OF the *Klebsiella pneumoniae*

M5a1 GENOME

by

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Klebsiella pneumoniae is an enteric opportunistic pathogen which commonly causes pulmonary, urinary tract and kidney infections. It has also been widely used for the study of nitrogen fixation. In this study, a macrorestriction map of *K. pneumoniae* M5a1 chromosomal DNA was constructed using pulsed field gel electrophoresis (PFGE) and Southern hybridization techniques. The genome DNA was digested with two rare cutting restriction enzymes (*Xba*I and *Bln*I) and the genome size was estimated from the sum of the fragment sizes as 5,121 Kb ($\pm$ 2.8 %). A tentative order of fragments was deduced using Southern hybridization with purified restriction fragments. Several auxotrophic mutants were isolated from M5a1 and the chromosomal clones that complemented them were identified. Random clones from the M5a1 chromosomal library were also obtained. These clones and clones acquired from other laboratories were mapped onto the circular macrorestriction map of M5a1. The order of fragments obtained by this means agreed with the map constructed by Southern hybridization of *Bln*I and *Xba*I fragments.

This map can be used to construct a typing system for clinical *K. pneumoniae* isolates based upon DNA fingerprinting. It will also facilitate the construction of a high resolution (microrestriction) map and the determination of the complete nucleotide sequence of the organism. Preliminary genomic analyses of clinical samples show that there are large variations in the genomic pattern of *K. pneumoniae* samples from different patients even though they are isolated from the same location of the body.

LOMA LINDA UNIVERSITY

Graduate School

A PHYSICAL MAP OF THE *Klebsiella pneumoniae* M5a1 GENOME

by

Alexandrine Randriamahefa

A Dissertation in Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biology

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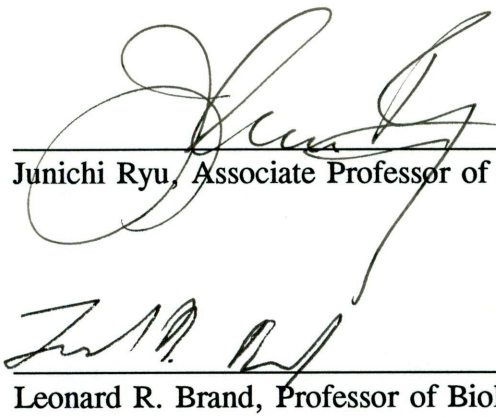

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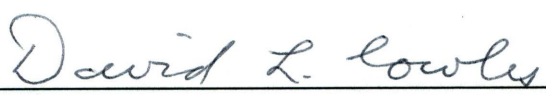

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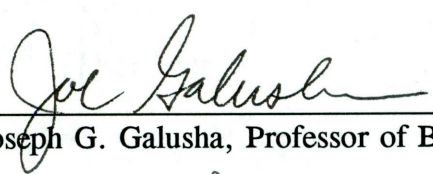

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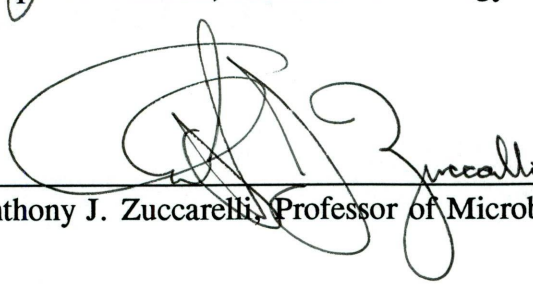

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INTRODUCTION

In 1882, Carl Friedlander isolated *Klebsiella pneumoniae (K. pneumoniae),* a gram negative encapsulated nonmotile rod, which is responsible for about five percent of human bacterial pneumonias (Davis, 1980). This organism can be found in the human intestine and in clinical specimens such as urine and sputum, as well as in soil and water. *K. pneumoniae* is present in the natural flora of humans, being found in 10 percent of all healthy adults and in 46 percent of strict vegetarians (Hentges, 1983). While four species of *Klebsiella* : *K. pneumoniae, K. ozaenae, K. oxytoca* and *K. rhinoscleromatis* are associated with human disease, *K. pneumoniae* is the only species commonly associated with pneumonia. It is clinically important to differentiate between pneumonia caused by *K. pneumoniae* and that caused by other other bacteria such as *Haemophilus pneumoniae* and *Streptococus pneumoniae* because *K. pneumoniae*, unlike *S. pneumoniae*, is resistant to penicillin. Secondly, *K. pneumoniae* tends to be more destructive to tissue, causing necrosis and abscess formation with irreversible lung changes.

This bacterium has been of clinical importance for several other reasons as well. It causes life-threatening bacteremia in adults (De la Torre et al., 1985) and in neonates (Morgan et al., 1984). In hospitals, *K. pneumoniae* is one of the major causes of septicemia in pediatric wards. It has been indicted as a pathogen in immune compromised individuals such as cancer patients, transplant patients and those suffering from congenital defects of the immune system (Baron, 1986).

K. pneumoniae is a prime suspect as the cause of pneumonia in alcoholics (Boyde and Hoerl, 1986).

A high percentage of *K. pneumoniae* strains from clinical isolates, particularly those from nosocomial infections, contain R-factors that confer resistance to a variety of drugs such as ß-lactams and sulfonamide (Vernet et al., 1992). All *Klebsiella* strains are ampicillin resistant and this resistance may be encoded by a gene present on the chromosome or on a plasmid.

Related bacterial species often possess similar proteins for essential physiological and metabolic functions (the so-called "housekeeping" functions), while at the same time displaying differences in surface components. Surface components which may vary among similar species include outer membrane proteins, flagellae, fimbriae and features of the lipopolysaccharide. A notable characteristic which distinguishes *Klebsiella* from most other bacteria in the family *Enterobacteriaceae* is that it forms large mucoid colonies due to the presence of a prominent polysaccharide capsule (K antigen). The presence of a capsule increases the virulence of this organism because it allows the cells to avoid phagocytosis. Although two distinct types of antigens are present in encapsulated strains of *Klebsiella,* one in the capsule and the other in the soma, serological typing is based on the K antigen from the capsular material. There are eighty serologically distinct capsules, some of which cross-react with the capsular polysaccharide from *S. pneumoniae* and from other species. The *rcs A* gene is involved in the expression of the K antigen capsule (McCallum and Whitfield, 1991). The gene product contributes to the pathogenicity of

the bacteria including the induction of immune tolerance (Nakashima et al., 1971) and impairment of maturation and function of macrophages (Yokochi et al., 1977).

Klebsiella strains possess two types of fimbriae (pili), one with a mannose-sensitive adhesin (type 1) and another with a mannose-resistant adhesin (type 3). *K. pneumoniae* was also reported to contain a proteinaceous endotoxin (Klipstein et al., 1977) which causes fluid accumulation in ileal loops. Some strains of *K. pneumoniae* produce a heat stable enterotoxin that induces hypersecretion of fluid and electrolytes into the small human intestine and gives rise to acute gastroenteritis.

Besides its pathogenic properties, *K. pneumoniae* is studied as a model system for nitrogen fixation. It is the first diazotroph (N_2-fixing bacterium) examined in a free living, non symbiotic condition (Kennedy, 1989). The diazotrophs contain nitrogenase, a group of complex enzymes that catalyze the conversion of N_2 to NH_3. Nitrogenase enzymes are rapidly inactivated by molecular O_2, hence most nitrogen fixing bacteria, even if aerobic, can fix nitrogen only when O_2 concentrations are low, while others such as *Azotobacter* possess complex mechanisms for maintaining low intracellular O_2 concentrations (Kennedy, 1989). *K. pneumoniae,* which are facultative anaerobes, can fix nitrogen only when O_2 is very low or absent. Most well known nitrogen fixing bacteria, in contrast to *K.pneumoniae*, live in symbiotic associations.

A study among the native tribes in the highlands of Papua New Guinea showed that the total nitrogen excreted in the feces and urine amounts to about twice that ingested by the inhabitants (Mitsuoka, 1978). In 1970, Bergersen and Hipsley

isolated nitrogen fixing *Klebsiella* and *Enterobacter* from the fecal bacteria of these individuals (Mitsuoka, 1978). Thus it was speculated that nitrogen fixing bacteria might synthesize protein from atmospheric nitrogen which enriches the intestine with a nitrogen source in addition to the food ingested. The transfer of fixed N_2 from NH_3 to human protein or amino acids has not been directly demonstrated but, if present, this may help the host to supplement the nitrogen uptake from the diet. If such transfer takes place, the high incidence of presence of *K. pneumoniae* in "strict vegetarians" (Hentges, 1983) may indicate that these individuals are substantially supplementing their protein intake through this symbiosis.

In the early 1970's, the bacterial genes necessary for synthesis of nitrogenase, (called *nif* genes) were found by conjugation and transduction methods to be next to the operon for histidine biosynthesis in *K.pneumoniae* (Streitcher et al., 1971). Subsequent studies showed that the 19 *nif* genes are arranged in a single chromosomal cluster but in eight transcriptional units and occupy a contiguous region of 24 Kb of DNA in the chromosome (Kennedy, 1989). The order of *nif* loci in symbiotic *Rhizobium* is largely the same as in *Klebsiella* despite the presence of these genes on a plasmid in *Rhizobium* and on the chromosome in *Klebsiella* (Krawieck and Riley, 1990). The genes are considered to be homologous due to positive DNA hybridization between the *nif* gene of *K. pneumoniae* and *Rhizobium*.

Although *K. pneumoniae* is important to both clinicians and basic scientists as a pathogen and as a model system for nitrogen fixation, the genetic study of this species lags behind compared to that of other enteric bacteria, for example, *E.coli* and

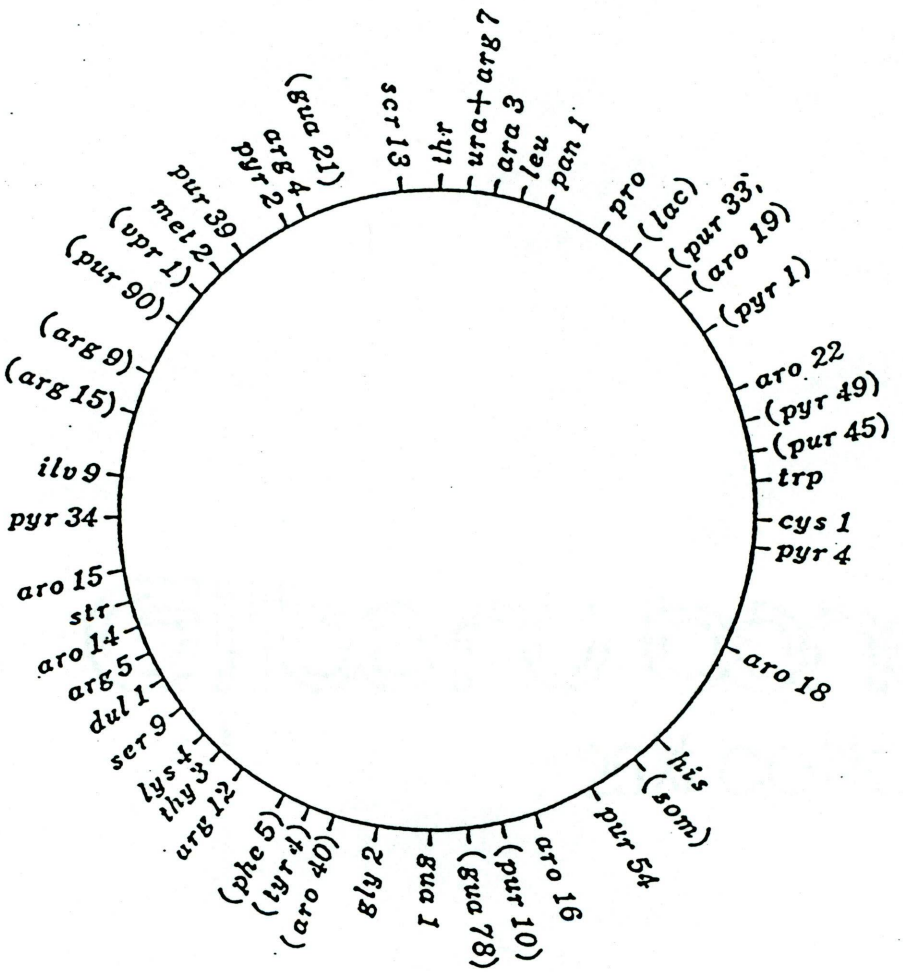
S. typhhimurium. Knowledge of the nature of the genome and the order of the genes on the chromosome of *K. pneumoniae* would be very useful to both areas of study.

The construction of a gene map is a fundamental step in the initial genetic characterization of an organism. Genome mapping was defined by Coulson and Sulston (1988) as "the generation of an ordered cloned library that fully represents a genome, together with enough genetic landmarks to allow accurate alignment of the physical and genetic maps". The first genetic linkage map was based on the process of homologous recombination. Crossing over between DNA segments results in an exchange of genes between the segments. The recombination frequency between two genetic loci is roughly proportional to the distance between them. In bacteria, homologous DNA segments can be brought into the same cell by conjugation, by transduction or by transformation. Linkages between bacterial genes when using this method for mapping, are expressed in terms of cotransformation frequencies or cotransduction frequencies. In the case of conjugation, the location of a gene on the chromosome is deduced from its earliest time of entry when mating is interrupted at various times. A genetic map summarizes the order of genes on the chromosome. Recent genetic maps of *E. coli* (Bachman, 1988) and *S. typhimurium* (Sanderson and Hurley, 1988) listed about 1400 and 700 genes, respectively, mapped onto their circular chromosomes. Among the gram positive bacteria, *Bacillus subtilus* 168 has the most extensively characterized genetic map with about 700 loci.

For *K. pneumoniae*, gene transfer can be accomplished either by conjugation (Dixon and Postgate, 1971), or by transduction with the bacteriophage P1 (Streicher et al., 1971 Kennedy, 1977). Matsumoto and Tazaki (1970) used conjugation to establish the first genetic linkage map of *K. pneumoniae* and mapped 28 genes which they found to be arranged circularly in a single linkage group. One year later (Matsumoto and Tazaki, 1971) genes coding for various aromatic acids (*aro*), pyrimidines (*pyr*), purines (*pur*) and other markers were added to the linkage map bringing the total number of genes to 49 (Fig.1).

In contrast to a genetic map, a physical map of a chromosome shows the structure of the DNA and specifies the actual distances in terms of base pairs between the cutting sites recognized by various restriction enzymes. Since the distances are measured in base pairs rather than in units of recombination frequency this map provides a direct and physical representation of the DNA molecule itself. Physical maps are much more precise in locating various features of the genome than are genetic maps and they are of superior utility for recombinant DNA techniques because they allow the location of various mutations and gene segments by their positions relative to restriction enzyme cleavage sites. The ultimate physical map, of course, would be the complete DNA sequence of the given organism. At present, however, complete DNA sequences for the entire genome are available only for a few *E. coli* phages such as ϕX174 (Sanger et al.,1977), lambda (Hendrix et al., 1983) and Mu (Symonds et al., 1987). Once the sequences of regions or all of the genome of a species are available, sequence comparisons can be made with other related species.

Figure 1. Genetic linkage map of *Klebsiella pneumoniae* from Matsumoto and Tazaki, 1971


pan 1
leu
ara 3
ura+ arg 7
thr
scr 13
(gua 21)
arg 4
pyr 2
pur 39
met 2
(vpr 1)
(pur 90)
(arg 9)
(arg 15)
ilv 9
pyr 34
aro 15
str
aro 14
arg 5
dul 1
ser 9
lys 4
thy 3
arg 12
(phe 5)
(tyr 4)
(aro 40)
gly 2
gua 1
(gua 78)
(pur 10)
aro 16
pur 54
his
(som)
aro 18
cys 1
pyr 4
trp
(pur 45)
(pyr 49)
aro 22
(pyr 1)
(aro 19)
(pur 33)
(lac)
pro

A comparative nucleotide sequence analysis between the two related bacteria, *E. coli*and *S. typhimurium*, showed a degree of similarity ranging from 77 to 100% for amino acid sequences and from 75 to 99% for nucleotide sequences (Riley and Sanderson, 1990).

Three technical advances have revolutionized the process of physical mapping. The first technique, introduced by Schwartz et al. (1984), reduces breakage to DNA during the isolation process. Most previous DNA extraction procedures have been carried out in free solution, where the DNA is subject to shear forces that are proportional to the square of its molecular weight (Cantor at al., 1988). In the new method the DNA is embedded in a protective agarose matrix that stabilizes the DNA molecules after the removal of all the membranes, proteins and RNA. This procedure makes it possible to isolate intact DNA molecules as large as 10 megabases.

The second technique allows the generation of large defined DNA fragments by using restriction endonucleases that cut the chromosome infrequently. The discovery of restriction endonucleases led to a quantum advance in genetic mapping and physical characterization of DNA. Before 1984, however, only a few restriction enzymes which cut DNA at low frequencies were available. These included two enzy-mes with eight-base recognition sites [*Not*I (GC/GGCCGC), *Sfi*I (GGCCNNNN/NGGCC)]. However, these enzymes cleave GC rich DNA frequently and may not cut AT rich DNA (McClelland et al.,1987). The isolation of more restriction enzymes with eight-base recognition sites [e.g *SgrA*I (C(A/G)/CCGG(T(C)G), *Pac*I (TTAAT/TAA), *Pme*I (GTTT/AAAC) and *Sse* 8387I

(CCTTGCA/ GG)] made it possible to cut chromosomal DNA from any source into large defined fragments.

There are other restriction enzymes which, though they have less than eight-base recognition site, can also be expected to cut certain genomes infrequently. The genomes of bacterial species vary widely in their base composition; G+C contents can range from 26% to 72%. McClelland et al. (1987) demonstrated that the relative abundances of certain DNA sequences in the genomes of bacteria have a profound effect on the frequency with which certain restriction endonuclease recognition sequences are found. For example, the tetranucleotide CTAG is extremely rare in most bacterial genomes with G+C contents above 45%. The extreme rarity of CTAG in bacteria may be caused by a bias in the repair of T-G mismatches (Wong and McClelland 1992, McClelland and Bhagwat, 1992). The CTAG sequence is thought to be a mutational "hot spot" in which the "T" of CTAG is misreplicated and paired with a "G" in the newly synthesized complementary strand. A special enzyme *vsr* (found in *E. coli*) acts to correct T/G mismatches. However, when repaired the "T" nucleotide is "corrected" to a 'C' nucleotide resulting in the change of the CTAG sequence to a CCAG. The frequent change of T to C leads to a decrease in frequency of the CTAG sequence in the genome. Accordingly, restriction endonucleases with cleavage specificities of six bases pairs containing the CTAG sequence cut the bacterial genome into only a few large DNA fragments that can then be resolved by pulsed field gel electrophoresis.

The third technique, pulsed field gel electrophoresis (PFGE) allows high resolution separation of DNA molecules ranging in size from 10 Kb to more than 15 Mb (Smith and Cantor, 1987). Conventional gel electrophoresis techniques for DNA analysis are effectively limited to molecules less than 50 Kb (Clark et al., 1988).

Above this size all DNA molecules have such similar mobilities in conventional electrophoresis that no separation can be achieved.

In the typical separation of DNA restriction fragments on conventional electrophoretic gels, differences in mobility are imposed by the sieving action of the gel matrix which results in molecules migrating at rates that are inversely proportional to the logarithm of their molecular weights; while the electrical field simply provides a unidirectional motive power. Very large DNA molecules can still enter the gel, but under the influence of the electric field they quickly align their long axis to that of the electric field. In this orientation they are able to move through the gel pores at a constant rate that is independent of their size in a manner called "reptation" (Levene and Zimm, 1989). Sieving is eliminated and the ability of ordinary gel electrophoresis to fractionate DNA molecules by size is lost. Early attempts to extend the range of separation to larger molecules relied on reducing the matrix concentration and using lower voltage gradients. Unfortunately, low percentage agarose gels (0.1%-0.5% agarose) are mechanically difficult to handle and the use of low voltage gradients requires run times of days to weeks. Therefore, to separate large DNA molecules in agarose gels, additional physical properties of DNA molecules have been

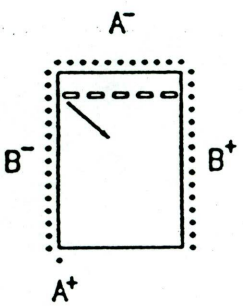
examined and exploited. Klotz and Zimm (1972) demonstrated that the viscoelastic relaxation time of large DNA in solution is related to its molecular weight. They further showed that the conformation of DNA molecules is perturbed by a voltage gradient and that the molecules return to the unperturbed state when the gradient is removed. Schwartz made use of these relaxation properties of large DNA molecules for their separation in agarose by using two alternating electric fields (Schwartz et al., 1982). As the direction of the electric field is changed periodically, reptating molecules are forced to relax from their stretched configuration and to reorient in the new field direction before they can continue migration. Smaller molecules will move faster than large molecules because they will be able to turn corners and reorient more quickly than can large molecules. The time required for this reorientation has been found to be very sensitive to molecular weight. Larger DNA must spend a large portion of the pulse time period reorienting before they begin to migrate through the gel and thus migrate less distance .

Representative apparatus for PFGE developed by various researchers are shown in Fig. 2. Schwartz and Cantor (1984) used alternating electric fields one homogeneous and the other non-homogeneous. The non-homogeneous field (non uniform) was achieved using an array of electrodes as the cathode and single point electrode as the anode (PFGE, in Fig. 2). Later that year, Carle and Olson (1984) used a similar apparatus, which was orthogonal but in which both the anode and the cathode were non homogeneous (OFAGE, in Fig. 2). The major drawback of this type of apparatus is that the electric fields are not uniform. This means that electric

Figure 2. Representative electrode geometries of different pulsed field systems. (1) Pulsed field gel electrophoresis, PFGE ; (2) Orthogonal field alternating gel electrophoresis, OFAGE ; (3) Transverse alternating field gel electrophoresis, TAFE ; (4) Field inversion gel electrophoresis, FIGE ; (5) Contour clamped homogenous electric fields, CHEF ; (6) Rotating gel electrophoresis, RGE .

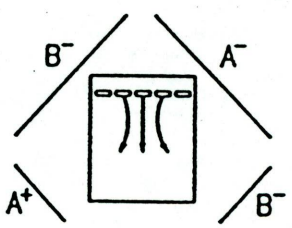
1

PFGE



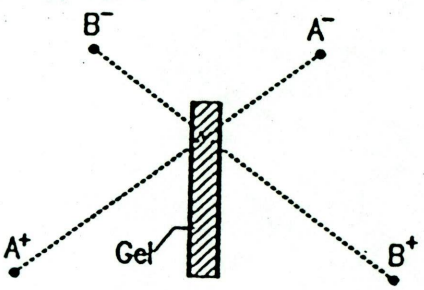
2

OFAGE



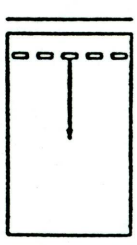
3

TAFE



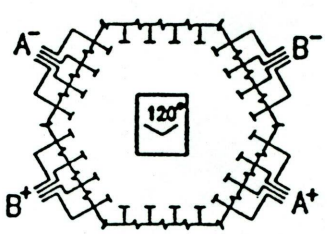
4

FIGE



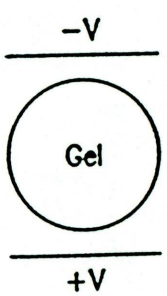
5

CHEF



6

RGE



field strength on individual lanes of migration are unequal so that DNA tracks run diagonally across the gel making accurate size estimation difficult. Gardiner et al.(1986) modified the system by orienting the electric field transversely to the gel(TAFE, in Fig. 2). Since the electric fields produced are homogenous across the width of the gel the bent lanes were eliminated, but with this design the molecules donot move at a constant velocity over the length of the gel because the angle between the electric field varies from 115° at the top of the gel to 165° at the bottom. Carle et al (1986) used simple electrode geometry by periodically inverting a uniform electric field in one dimension causing a 180° angle of reorientation. This system is called field inversion gel electrophoresis (FIGE, in Fig. 2). Although simple, the FIGE configuration assures that at any given switch time, DNA molecules of different sizes may exhibit the same mobility and give a band inversion. Chu et al. (1986) generated homogeneous electric fields using multiple electrodes arranged around a closed contour. An array of electrodes were arranged in a hexagonal contour which offered reorientation angles of 60°- 120° (CHEF, in Fig. 2) resulting in straight lanes using angle reorientation. Another system, called rotating gel electrophoresis (RGE, in Fig. 2) uses a single homogeneous field and changes the orientation of the electric field in relation to the gel by discontinuously and periodically rotating the gel.

Good resolution of DNA fragments depends on many electrophoretic conditions, such as field shape, voltage, pulse time, agarose concentration and temperature. These pulsed field parameters are interdependent. Maximal speed and resolution are the common goals in selecting pulsed field gel conditions for any

particular separation. Most of the changes which alter the rate of migration also alter the resolution in the size range of the separation.

The development of PFGE has opened up many new possibilities in molecular genetics. Because PFGE allows the rapid mapping of genomic regions that are hundreds of Kb long, the technique can be used both to study the molecular organization and to sequence the genome of different organisms. See the appendix for a list of various features of many bacterial genomes that have been studied by PFGE and the two other new techniques. *Mycoplasma genitalium* has the smallest known chromosome of only 585 Kb, while *Myxococcus xanthus* has the largest known bacterial chromosome of 9,454 Kb. *Rhodobacter sphaeroides* 2.4.1 was found to have two chromosomes of 3,046 and 914 Kb. *Borrelia burgdorferi* B31 and *Streptomyces lividans* 66 have linear forms of genomic DNA. A unique DNA arrangement is found in *Archaebacterium haloferax volcanic* DS2 in which the cell has one chromosome of 2,920 Kb and four other plasmids.

Our laboratory has previously identified and cloned a new restriction modification system in *K. pneumoniae* M5a1, designated KpnAI (Valinluck, 1992). As a step in the further characterization of this strain I undertook to determine the physical length of the chromosome of M5a1. Previously, the size of bacterial chromosomes has been estimated using several different methods: (i) kinetics of renaturation (Gillis et al., 1970), (ii) two-dimensional gel electrophoresis of restriction fragments (Yee & Inouyl, 1982), (iii) electron microscopy techniques (Chow, 1977) and (iv) summing the sizes of large restriction fragments after PFGE (Smith et al., 1987). In the last

case the genome was digested with a restriction endonuclease to produce a small number of large fragments which were then separated by pulsed field gel electrophoresis. The sum of the lengths of these fragments yielded the size of the genome.

The second goal of this work was to order the fragments from total genomic DNA digested with restriction endonucleases to construct a physical map. The order of the fragments was deduced from Southern hybridization.

The third goal was to confirm the map by using known and random clones to identify the orientation of the physical map. The clones used to confirm the map were of three types: (i) known clones from other laboratories, (ii) clones which complemented auxotrophic mutations and (iii) random clones from a genomic library pool.

The fourth goal was to identify polymorphism in the genomic DNA of clinical strains of *K. pneumoniae* using a procedure known as DNA fingerprinting. The restriction patterns of genomic DNA from different strains were compared to each other and to the reference DNA pattern constructed here. This approach allowed sensitive differentiation among the strains based upon minor variations in their restriction genomic patterns.

MATERIALS AND METHODS

Materials

A. Bacterial strains

K. pneumoniae M5a1 was obtained from L. Bullas, Department of Microbiology, Loma Linda University, Loma Linda, CA. This strain was originally obtained from C. Kennedy of the University of Sussex, England.

All bacterial strains were isolated on L-agar plates (Silhavy et al., 1984) or grown in liquid culture in L-broth. Overnight cultures were prepared by inoculating 0.5 ml of culture into 10 ml L-broth (20-fold dilution) and incubating overnight without shaking, or by inoculating one colony from an agar-plate into 10 ml of L-broth.

For all experiments, fresh cultures were prepared by inoculating 0.1ml of overnight culture into 10 ml of L-broth and incubating them at 37°C on a rotator (Techni Lab instruments, Inc., Pequannock, N.J.) to mid-log or log phase as determined by growth curve.

Permanent stocks of new mutant strains were made by adding two drops of dimethylsulfoxide (DMSO) to 1.5 ml of bacterial culture and freezing at -80°C.

B. Media

L-broth (Silhavy et al., 1984) consisted of 10 g of Bacto Tryptone (Difco, Detroit, MI), 5 g of Yeast Extract (Difco), 5 g of NaCl and 1 g of dextrose per liter of water. Bacto Agar (15 g) was added for L-agar plates.

Davis minimal medium contained 875 ml of water, 15 ml of 20% (wt/vol) glucose (final concentration 0.3% [wt/vol]) and 100 ml of Davis salts solution (70 g of K_2HPO_4, 20 g of KH_2PO_4, 5 g of sodium citrate, 10g of $[NH_4]_2SO_4$ and 1g of $MgSO_4$ per liter of water). When required, amino acids and vitamins were added to final concentrations of 20 μg/ml and 1 μg/ml, respectively.

SOC medium (Bio Rad laboratories, Richmond, CA) was used for electroporation and consisted of 2% Bacto Tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

All media were sterilized before use by autoclaving at 120°C and 15 psi for 20 to 45 min. Sterilized media were cooled to 50°C to 55°C before the addition of antibiotics or other chemicals.

Antibiotic solutions used as addition to L-agar or L-broth were made with distilled water, except for chloramphenicol, which was dissolved in 75% ethanol. The final concentration was 1000 μg/ml for ampicillin and 180 μg/ml for chloramphenicol. Antibiotics were sterilized by passing through a 0.45 μm HA filter (Millipore, Bedford, MS). The stock solution were stored at 4°C and used within two weeks.

C. Buffers

1. Bacterial buffers

A phosphate buffer (B-buffer) (Silhavy et al., 1984) was used for bacterial dilution. It consists of 3 g of KH_2PO_4, 7 g of NaH_2PO_4, 4 g of NaCl and 0.2 g of $MgSO_4$ per liter (pH 7.0).

2. Buffers for DNA analysis

a. Buffer for pulsed field gel electrophoresis.

One liter of EC lysis buffer was made with 6 ml of 1.0M Tris-HCl (pH 7.6), 200 ml of 5 M NaCl, 200 ml of 0.5 M EDTA, ethylenediamine-tetraacetate (pH 8.0), 50 ml of 10% of Brij-58, 5 ml of 4% of deoxycholate, 2.5 ml of 20% of N-lauroylsarcosine. The stock was stored at -20°C and 1 mg/ml lysozyme and 20 μg/ml RNase were added just before use. RNase was incubated for 20 min at -80°C before use.

One liter of Pett IV made with 10 ml of 1 M Tris-HCl, pH 7.6 and 200 ml of 5 M NaCl, was autoclaved and stored at room temperature and used to wash the bacterial cells.

ES buffer (100 ml) was made with 95 ml of 0.5 M EDTA, pH 8.0 and 5 ml of 20% of lauroylsarcosine. To make ESP buffer, 1 mg/ml of Proteinase K (Sigma) was added to ES buffer, incubated for 2 hr at 37°C and stored at 20°C.

Tris-EDTA (TE) buffer (pH 7.5) consisting of 10 mM Tris-HCl and 1 mM EDTA, was autoclaved and used to dissolve DNA. Phenylmethylsulfonyl fluoride

(PMSF) (final concentration 1.0 mM) was added when it was necessary. PMSF stock (final concentration 100 mM) was prepared in isopropyl alcohol and used fresh.

Tris-borate EDTA buffer (TBE, pH 8.0, 10x) contained 108 g Tris base, 55 g boric acid and 7.4 g EDTA in one liter of water and was diluted to 0.5x with distilled water for gel electrophoresis.

b. Buffer for mini-gel electrophoresis

The stock solution of 40x Tris-acetate EDTA (TAE) buffer (Davis et al., 1986) consisted of 193.6 g of Tris base (1.6 M), 65.6 g of anhydrous sodium acetate (0.8 M) and 13.5 g of anhydrous EDTA-Na_2 (40 mM) per liter. To adjust the pH to 7.2, 50 ml of 12.1 M (37.2%) HCl was added. To stain the DNA, 25 μl of 10 mg/ml ethidium bromide was added to one liter of 1x TAE for gel electrophoresis buffer.

The 6x loading buffer was made with 0.25% bromophenol blue and 30% glycerol in water for visualizing DNA migration on agarose gel electrophoresis.

The 5x stopping buffer for restriction enzyme consisted of 0.25% bromophenol blue, 25% glycerol and 100 mM EDTA.

3. Buffer for DNA hybridization.

Two different hybridization buffers were used for the study. The first was modified from Maniatis et al. (1982) and the second was commercially available from Amersham International (Arlington Height, IL) and used according to the recommendation of the manufacturer.

The prehybridization solution (2.5x) was made with 0.25% sodium dodecyl sulfate (SDS), 0.25% Ficoll (Sigma chemical Co.,St.Louis, MO), 0.25% polyvinyl pyrolidone (PVP), 0.25% bovine serum albumin, 2.0 M NaCl, 0.25 M piperazine-N,N'-bis[2 ethanesulfonic acid] (PIPES),pH 7.0 and 500 μg/ml salmon sperm DNA.

The Ficoll, PIPES, PVP and SDS, were boiled together and then NaCl was added. After the mixture had cooled to room temperature bovine serum albumin was added. Salmon sperm DNA was sheared with a syringe needle, boiled for a few minutes and then added to the first solution. The solution was stored at -20°C.

The prehybridization solution (1x) consisted of a 2:2:1 mixture of formamide, 2.5x prehybridization solution and water (Maniatis et al., 1982; p.447).

The 20x SSC solution consisted of 88.2 g of sodium citrate and 175.2 g of NaCl per liter, the pH of which was adjusted to 7.0 with concentrated HCl.

The low stringency buffer contained 2x SSC and 0.1% sodium dodecyl sulfate (SDS). The high stringency buffers were 1x SSC and 0.1% SDS; 0.7X SSC and 0.1% SDS.

DNA denaturing buffer was made of 1.5 M NaCl and 0.5 M NaOH per liter of water.

DNA neutralizing buffer consisted of 0.5 M Tris base and 1.5 M NaCl per liter, the pH of which was adjusted to 8.0 with concentrated HCl.

Methods

A. Growth curve for M5a1.

A 10 ml culture in L-broth was begun by inoculating 0.5 ml of an overnight culture and incubating at 37°C in a rotary shaker. Growth was monitored every 30 minutes by measuring the OD_{510} with a spectrophotometer. Every 30 minutes dilutions to 10^{-4} and 10^{-5} were made in B buffer and 100 μl from each of these dilution were plated on L-agar. Plates were incubated at 37°C overnight and the colonies were counted to generate a growth curve.

B. Chromosomal DNA isolation

1. Isolation of bacterial genomic DNA using the G-NOME™ DNA kit (BIO 101, Inc.).

Five ml of overnight cell culture was centrifuged for 5 min at 10,000 rpm in a Sorvall SS-34 rotor and the pellet was resuspended in 2 ml of cell Suspension Solution (as provided by the kit). Subsequent solutions in this method are all those provided in the kit. RNase mix (55 μl) and cell Lysis/Denaturation solution (35 μl) were added and mixed thoroughly by inversion and the mixture was incubated at 55°C for 15 min. Then 175 ml of 20x Protease Salts and 30 ml of Protease Mix were added and the sample was incubated at 55°C for 30 min. This was followed by the addition of 1 ml of "Saltout Mixture" and the mixture was centrifuged in an IEC HN-SII table top centrifuge (Damon, IEC division, Needham Heights, MA) at 2,500

rpm for 2 min. The supernatant was transferred to a clear plastic tube and 50 μl of spooling salts and 6 ml of 100% ethanol were added. Threads of DNA were spooled onto a glass rod, air dried to eliminate excess ethanol and dissolved in 300-500 μl of TE.

2. DNA extraction with phenol/chloroform.

Cultures were grown overnight in 10ml of L-broth and centrifuged at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet was resuspended in 1 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA), transferred to a 15 ml Corning polypropylene centrifuge tube and frozen at -20°C for at least 30 min or at -80°C for 5 min. When just thawed, 100 μl of 10 mg/ml lysozyme (in 0.25 M Tris-HCl, pH 8.0) were added and the cells were incubated on ice for 45 min. 1 ml of extraction buffer was then added followed by the addition of 200 ml of 10% SDS (1% final concentration). The mixture was incubated at 65°C in a waterbath for 20 min, then 25 ml of Proteinase K (10 mg/ml) was added and the mixture was incubated in a 65°C waterbath for 2 hr or at 37°C overnight. An extraction with equal volumes of phenol saturated with Tris-HCl (pH 8.0) was performed. The mixture was centrifuged in a IEC HN-SII table-top centrifuge at 2,500 rpm for 5 min. After a final extraction with an equal volume of chloroform/isoamyl alcohol (24:1) the aqueous layer was transferred to a sterile 15 ml Oakridge centrifuge tube and 50 μl of RNase (5 mg/ml) were added and incubated at 37°C overnight. Three more extractions were performed as described above and the final aqueous layer was transferred into a new 15 ml Oakridge tube and mixed with

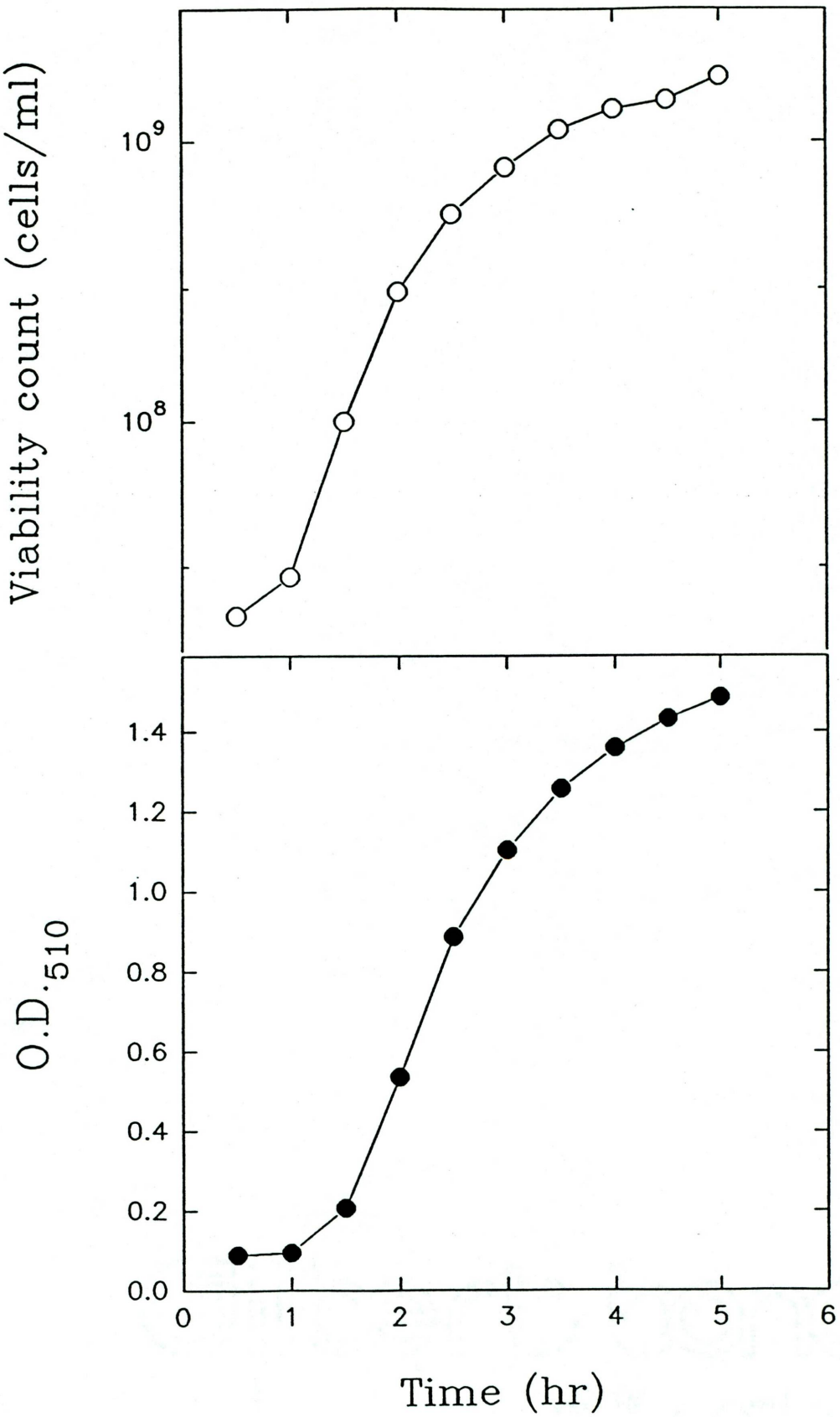
1/10 volume of 3.0 M Na-acetate (pH 5.2) and 2 volumes of cold 100% ethanol. The mixture was gently inverted, incubated at -80°C for 10 min and centrifuged at 10,000 rpm in a Sorval SS-34 rotor for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The DNA was then dried in a Speed-Vac centrifuge for 10 min and finally dissolved in 500 μl of TE buffer and refrigerated at 4°C overnight.

3. Preparation of chromosome DNA for PFGE

One of the major variables in applying pulsed field gel electrophoresis to DNA from a new organism is to determine the appropriate amount of DNA to load onto a gel in order to get good resolution. The best result is generally obtained with a small amount of DNA usually between about 0.1 to 2.5 μg per sample (Smith, 1990).

The relationship between the optical density and the number of bacteria varies from strain to strain, thus a growth curve which relates the number of viable bacteria to the optical density OD_{510} for M5a1 was constructed (Fig.3). At an OD of 0.2 the culture contained 10^8 cells/ml. It is difficult to determine the exact DNA concentration because of the variation of the efficiency of the preparation, but relative DNA concentration can be estimated, by applying the calculation proposed by Birren and Lai (1993), for any organism. Taking *E. coli* as a reference organism and its known chromosome size of 4.7×10^6 base pairs, the following calculation was made.

Figure 3. *K. pneumoniae* M5a1 growth curve. Cells were grown at 37°C and rotated. Panel A: viable cells cells/ml. Panel B: optical density at 510 nm.


Viability count (cells/ml)
10^9
10^8
O.D.$_{510}$
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time (hr)

A culture of 10^8 CFU /ml will yield :

10^8 x 4.7 x 10^6 DNA base pairs x 660 g/mole DNA base pairs/

6 x 10^{23} molecules per mole= 0.517 μg/ml.

According to this formula, I made the following calculations: 10 ml of culture will produce 5 μg of *K. pneumoniae* genomic DNA in 1 ml. When 100 μl of the culture was used to make one agarose gel plug and about 1/6 of the plug is loaded into a single well, the final amount was approximately 0.1 μg of DNA. The highest quality DNA was obtained from cultures in the mid-log phase of growth.

A fresh culture was grown to log-phase containing about 10^8 cells/ml (OD_{510} of approximately 0.2). Chloramphenicol (final concentration 180 μg/ml) was added to synchronize DNA replication forks at the origin of replication. Cells were chilled on ice and pelleted at 8,000 rpm in a Sorvall SS-34 rotor at 4°C for 15 min. The pellet was washed in 10 ml of Pett IV and centrifuged again at 8,000 rpm. The pellet was suspended in enough Pett IV to produce twice the DNA concentration needed for the final gel plugs. Then the cells were warmed to 37°C and diluted with an equal volume of 1% liquid Incert agarose. A 100 μl volume of the cell-agarose mixture was aliquoted into a plug-forming mold, then cooled at - 20°C for 10 min to solidify the gel. Gel plugs were ejected from the mold into a tube containing 2 volumes of EC-lysis buffer and incubated for 16 hr with gentle shaking to lyse the cells. The plugs were suspended in ESP solution for 48 hr with two exchanges of the buffer to digest bacterial proteins. To inactivate the proteinase K, the inserts were incubated in TE containing PMSF overnight at 37 °C with an exchange of the PMSF reagent after

two hours. Samples were washed with TE buffer for 2 hrs and then incubated in 20-40 units/μg DNA of restriction endonuclease digestion overnight. The restriction digestion was stopped by aspirating the mixture and treating the plugs with ES and ESP to remove the restriction enzyme. About one-sixth of plug was sliced off and loaded into a PFGE well. DNA prepared in this manner can then be stored at -4°C for one year (Smith L.C., 1990). Lambda ladders (New England Biolabs Inc, Beverly, MA), *Saccharomyces cerevisiae* chromosomes (New England Biolabs Inc, Beverly, MA) and a 5 Kb ladder (BioRAD, Richmond, CA) were used as size markers in different experiments depending upon the target size range of the unknown DNA molecules.

C. Plasmid DNA isolation

Two different protocols for plasmid preparation, the large scale plasmid preparation and the mini-prep (Promega) were used in this study.

1. Large scale plasmid preparation.

The cells from 1.5 ml of overnight culture were pelleted by 1 minute of centrifugation and resupended in 100 μl of lysis solution containig 5 mg/ml lysozyme, 9 mg/ml glucose, 10 mM EDTA and 110 mM Tris-HCl, pH 8.0, vortexed and incubated on ice for 30 min. Following the addition of 200 μl of extraction buffer (0.2 M NaOH and 1% SDS), the mixture was kept on ice for 5 min. Finally, 0.15 ml of 3 M sodium acetate (pH 4.9) was added and the solution was incubated on ice for 30 more min. The mixture was then centrifuged for 10 min at 4°C and the DNA

was precipitated from the superatant at -80°C for 10 min with 2 volumes of cold 95% ethanol. After microcentrifugation at 4°C for 15 min, the DNA was rinsed in 0.5 ml of 70% ethanol, dried in a Speed-vac concentrator (Savant, Farmingdale, NY) and dissolved in 20 μl of TE buffer.

2. Plasmid isolation using Magic™ Minipreps DNA purification system kit (Promega, Madison, WI)

Three milliliters of overnight culture were pelleted for 5 min in a table-top centrifuge and the cells were resuspended in 200 μl Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNaseA). The cells were lysed with the addition of Lysis Solution (0.2 M NaOH and 1% SDS). The mixture was centrifuged for 5 min and the cleaned supernatant was transferred to a clean microcentrifuge tube with 1 ml of Purification Resin. The Resin was rinsed with 2 ml of Wash Solution in a minicolumn made from a disposable syringe barrel. The column was dried by centrifugation in a table-top centrifuge for 20 sec and then the DNA was eluted with 50 μl TE or water and a 20 sec centrifugation to extract the liquid.

D. Restriction endonuclease digestion.

For mini-gel electrophoresis and screening plasmid DNA, reaction mixtures were prepared with 1 to 8 μl of plasmid DNA, 1 μl of 10x enzyme reaction buffer, 1 μl of restriction enzyme and sufficient water or TE to make a total volume of 10 μl. The mixture was incubated at 37°C for at least 1 hr, followed by 10 min at 65°C to

inactivate the enzyme, then 2 μl of 5x stopping buffer was added. To digest DNA in 50 μl of agarose plugs for pulsed field gel electrophoresis, 50 ml reaction mixtures were prepared with 10 ml of 10x enzyme reaction buffer, 20-40 units of enzyme (*Bln*I and *Xba*I) and water to make up the total volume. Plugs were incubated in the mixture at 37°C overnight.

E. Isolation of DNA from agarose gels.

DNA was extracted from the agarose gels using one of the following three methods.

1. Geneclean kit (Bio 101, La Jolla, CA).

For small DNA fragments less than 100 Kb separated in 1.2% Seakem GTG agarose, the Geneclean Kit was used. Each DNA band was excised from the agarose gel with a razor blade and up to 0.4 g was transferred to a 1.5 ml micro-centrifuge tube. Three volumes of saturated sodium iodine were added and the mixture was incubated at 45°C to 55°C for 5 min to dissolve the agarose; then 5 μl of the glassmilk suspension was added and the mixture was incubated on ice for 5 min to allow binding of the DNA to the glassmilk beads, mixing every 1-2 min to keep the glassmilk in suspension. The mixture was spun for 5 sec and the super-natant was discarded, then the pellet was washed three times with 500 μl volumes of New Wash. After the last wash, the DNA/glassmilk was centrifuged twice to remove the wash solution. The DNA was eluted from the glassmilk pellet by incubating

it for 2-3 min with 5 to 10 μl TE buffer at 45-55^{0}C. Then the mixture was pelleted again and the supernatant containing the DNA was transferred to a new tube.

2. Elu-Quick kit (Schleicher & Schuell, Inc., Keene, NH)

For DNA fragments larger than 100 Kb the ELU-QUICK kit was used. DNA bands in 1.2% GTG agarose were suspended in 3.8 volumes of Binding Buffer and incubated at 50°C for 5 min. Then 20 μl of glassmilk concentrate was added and the mixture was kept at room temperature for 10 min then the tube was inverted for one minute and was centrifuged at 5,000 rpm for 30 seconds. The pellet was resuspended twice in 500 μl of wash buffer and pelleted again at 5,000 rpm for 30 sec. The pellet was resuspended in 500 μl of Salt Reduction buffer and centrifuged for 2 min at 5,000 rpm and the supernatant was discarded. Then the pellet was dried, the DNA was eluted with 30 ml of sterilized water or TE buffer and incubated at 50°C for 5 min. DNA was centrifuged for 30 sec at 5000 rpm and the supernatant containing the isolated DNA was transferred to a new tube.

3. Magic PCR™ preps kit for low-melting agarose gel.

A DNA band excised from a 1% low melting agarose, was transferred to a microcentrifuge tube and incubated at 70°C until it was completely melted. One ml of Resin was then added and the mixture was vortexed for 20 sec. The Resin was placed in a minicolumn, washed with 2 ml of 80% isopropanol and centrifuged for 20 sec at 5000 rpm to dry the Resin. The DNA was eluted by melting the Resin with 50 μl TE and centrifuging 20 sec at 500 rpm to extract the liquid.

F. Gel electrophoresis.

Two different gel electrophoresis apparatus were used to separate DNA fragments.

1. Minigel apparatus.

Minigels were used to separate and quantify small amounts of DNA fragments. The gel contained 0.8% Seakem GTG agarose and 1xTAE buffer containing 0.25 μg/ml of ethidium bromide. They were run for 1-3 hr at 30-50 volts in an electrophoresis apparatus (Mini Submarine Agarose Gel Unit Model HE 33, Hoeffer Scientific Instruments, San Francisco, CA) containing 250 ml of 1x TAE buffer with ethidium bromide. Gels were photographed on a Foto/Phoresis I unit (Fotodyne Inc., New Berlin, WI) with ultraviolet light (300 nm) on Polaroid film 667 (Cambridge, MA) exposed for one sec at f/4.5 to f/8. The migration distances were measured with a fluorescent ruler.

2. Pulsed field gel electrophoresis.

A LKB 215 Pulsaphor unit and a HEX electrode kit [(CHEF) Pharmacia LKB Biotechnology, Bromma, Sweden] were used to separate large DNA fragments embedded in the agarose gel. Gels contained 1.32 g of Seakem GTG agarose in 110 ml of 0.5x TBE, poured into a 15x15 cm frame. Slices of samples containing 0.1 to 0.5 μg of DNA in agarose blocks and DNA markers were loaded into the wells and sealed with agarose. Gels were run at 6-10 V/cm, 170-200 mA in a 0.5x TBE at 9°C. The pulse times were adjusted from 1 sec to 100 sec to provide optimal separation of fragments in each particular size range. Then the gels were stained in

0.5x TBE containing 0.5 μg/ml for 20-30 min and photographed on a UVPTM-36 transilluminator (UVP, Inc., San Gabriel, CA) using Polaroid 667 film exposed for one second at f/5.6 or f/8. The gel was destained in 0.5x TBE for 3 hr before Southern blotting.

G. Determination of the DNA concentration.

The concentration of DNA samples was estimated by comparing their fluorescence after electrophoresis in agarose minigels stained with 0.25 μg/ml ethidium bromide with samples of known concentrations. Two standards were used: 200 ng of a 1 Kb DNA ladder and a λ Hind III digest with DNA concentration of 327 μg/ml determined by absorbance at 260 nm. The A_{260} of double-standed DNA to 50 μg/ml was assumed to be 1.0 (Maniatis et al., 1982).

H. DNA-DNA hybridization

1. Southern blotting

After the destaining, pulsed field gels were exposed to the UVPTM-36 transilluminator for 5 min and depurinated in 400 ml 0.25 M HCl on an orbital shaker (Bellco Biotechnology) for 30 min. The gels were transferred to 400 ml of denaturation buffer, shaken for 1 hr, rinsed with deionized water and neutralized with neutralizing buffer for 1.5 hr on the shaker. DNA was transferred from the agarose gel to a Hybond-N nylon membrane (Amersham Co., 0.45 μm mesh) for multiple probings or to a nitrocellulose membrane (Schleicher and Schuell, 0.45 μm mesh)

using the Southern transfer method (Southern, 1975, Maniatis et al.,1982). The stack contained, in order, a glass plate, a filter paper kept continuously wetted with 20x SSC, the agarose gel, a nylon membrane cut to the size of the gel, a mask of plastic wrap to keep the edges of the gel from drying and one sheet of gel blot paper (GB 003, Schleicher and Schuell).

After blotting for two days, the membrane was removed, the position of the wells were marked and the DNA was bound to the membrane using the Stratalinker UV Crosslinker (Stratagene, La Jolla, CA). The membrane was cut into strips containing individual wells for use in hybridization to different probes.

2. Prehybridization

Two different procedures were performed.

a. Prehybridization. The 1x prehybridization solution was heated for 10 min in boiling water to denature the DNA, quickly chilled in ice water and poured into a glass Hyb-Aid bottle (National Labnet Co.,) containing the nylon membrane between nylon mesh sheets. The bottle was rotated in the Hyb-Aid oven for 6 to 16 hr at 42°C. The prehybridization mixture was removed and stored at room temperature for later use.

b. The membrane was incubated for 2 hr at 65°C in rapid prehybridization solution (Amersham Co.).

3. DNA hybridization

A ^{32}P-labeled DNA probe was heated for 10 min in a boiling water bath to denature the DNA. After being quickly chilled in ice water, the solution was poured

into the bottle containing the membrane. The bottle was rotated overnight at 42°C in the Hyb-Aid oven. To remove excess unbound probe, the membrane was rinsed thoroughly with 1x low stringency buffer, then washed twice for 30 min with 50 ml of 1x low stringency buffer at 55°C and twice for 30 min each time at 55°C with 50 ml of 1x high stringency buffer.

If the rapid hybridization buffer Amersham Co. was used, the membrane was washed twice in 2x SSC, 0.1% SDS for 10 min at room temperature, followed by 15 min at 65°C in 1x SSC, 0.1% SDS and two final washes in 0.7x SSC, 0.1%SDS at 65°C for 15 min. The washed membrane was put between two sheets of plastic wrap and placed in a Kodak X-omatic autoradiograph cassette with double-sided Kodak Lanex fine intensifying screens (Eastman Kodak Co., Rochester, NY). Static was removed using the Anti-Static Instrument Zerostat 3 (Discwasher, Columbia, MO). The membrane was then autoradiographed for 1 hr to 2 days at -80°C using Kodak OMAT XAR-5. Films were developed in a Konica medical film processor QX-70.

4. Dehybridization

To remove all the bound probe DNA, Hybond-N membrane strips were soaked in 0.4 M NaOH at 50°C for 30 min and incubated in a solution of 0.1x SSC, 0.1% SDS and 0.2 M Tris-HCl pH 7.5 for 30 min at 50°C; Nitrocellulose membranes were boiled in 0.1% SDS for 30 to 60 min. To verify complete removal of probes, deprobed membranes were exposed to X-ray film. Strips of nylon membrane were probed up to five times.

I. DNA labelling

A DNA labelling kit (Megaprime Amersham Co.) was used to prepare probes. Each reaction contained 2.5 - 25 ng of DNA. The DNA was heated for 5 min in a boiling water bath, then chilled and collected at the bottom of the microcentrifuge tube by spining for 1 sec. Then, 4 μl each of the unlabelled nucleotide solutions (dATP, dGTP, dTTP), 5 μl of reaction buffer, 5 μl of [α-^{32}P] dCTP (3000 mCi/mmol) (DuPont, Wilmington, DE) and 2 μl of Klenow DNA polymerase were added and mixed gently by pipetting up and down. The mixture was incubated at 37°C for 10 to 30 min. Unincorporated nuleotides were removed from the labelled DNA using a Super Select D mini-spin column (5 Prime- 3 Prime, Boulder, CO) containing Sephadex G-50. The column was precentrifuged for 4 min at 2,500 rpm in a table-top centrifuge to remove excess liquid. The reaction mixture was loaded on top of the column and centrifuged for 5 min at 2,500 rpm. The eluate containing the labelled DNA and a sample was counted in a liquid scintillation counter. The labelled reaction mixture was diluted to approximately $3x10^6$ cpm/ml in the prehybridization buffer.

J. Cloning bacterial genes which complement the nutritional requirement of M5a1.

1. Construction of plasmid libraries of M5a1 chromosomal DNA.

Chromosomal DNA of M5a1 extracted as described above was dissolved in TE buffer to a final concentration of 50 ng/μl and partially digested with *Sau*3AI

restriction endonuclease. Serial dilutions mixtures (1, 1/3, 1/9, 1/27 and 1/54) were made in five different tubes. A reaction mixture of 89 μl of genomic DNA, 1μl of 100x of bovine serum albumin (BSA) and 10 μl of *Sau*3AI reaction buffer was divided into the five tubes described above (30, 20, 20, 20, and 10 μl). Five microliters of *Sau*3AI (25 units) were added to the mixture of the first tube and mixed thoroughly, then 10 μl of the mixture were transferred into the second tube and the same dilution was carried out up to the fifth tube. The tubes were incubated at 37°C for 15 min and then at 65°C for 10 min to stop the reaction. One microliter of each reaction was run on a 0.8% agarose gel at 35 volts for 2 hr to check the partial digestion. The 1/27 and 1/54 diluted reaction mixtures contained the fragments of DNA in the 3 to 12 Kb range. These two dilutions were pooled together and run on a 0.8% agarose gel. DNA fragments between 3 Kb to 12 Kb were excised from the gel and recovered using the Geneclean method.

pUC18 plasmid DNA linearized with *Bam*HI has termini that can anneal and ligate to *Sau*3AI. A ligation reaction was prepared with a 3 to 1 ratio of *Sau*3AI M5a1 fragments to vector. The reaction mixture contained 45 μl (about 300 ng) *Sau*3AI fragments, 3 μl (100 ng) dephosphorylated pUC18 linears, 6.5μl ligation buffer, 6 units T4 DNA ligase (Promega) and distilled water to make a total volume of 65 μl. To check the completeness of dephosphorylation, a duplicate reaction but *Sau*3AI fragments was carried out. The mixtures were incubated at 16°C overnight. The reaction products were then extracted once with phenol/chloroform and once with chloroform alone and the DNA in the aqueous phase was precipitated by adding one-

tenth volume of cold 3 M sodium acetate (pH 5.2) and two volumes of cold absolute ethanol. After cooling at -20°C for 30 min the DNA was pelleted by centrifugation for 10 min at 4°C in a microcentrifuge, rinsed twice with 70% ethanol, dried in a vacuum and dissolved in 10 μl of water for use in transformation.

2. Preparation of competent cells for electroporation.

Competent cells were prepared by the procedure developed by Bio Rad Laboratories (Richmond, CA). One milliliter of overnight culture was inoculated into 100 ml of L-broth and incubated at 37°C with vigorous shaking until the OD_{600} reached 0.5 to 0.8. The culture was chilled on ice for 15 to 30 min and centrifuged in a cold GSA rotor at 5,000 rpm for 15 min. The pellet was washed twice with cold water (100 ml) and once with 10 ml of 10% glycerol. Finally, the cells were suspended in 2 to 3 ml of 10% glycerol to a final concentration of at least 3 X 10^{10} cells/ml. Competent cells were dispensed in 40 μl aliquots and stored at -80°C.

3. Electroporation.

The pUC18 library of *Sau*3AI fragments was first amplified in *Escherichia coli* XL1-Blue in order to maximize the size of the plasmid pool. A volume of 1μl to 3 μl of the DNA concentrated from the ligation reaction was added into six 40 μl aliquots of competent XL1-Blue cells. The mixture was incubated on ice approximately 1 min and then transferred to a cold 0.2 cm electroporation cuvette. Electroporation was performed using the Bio-Rad Gene pulser (Bio-Rad laboratories) (Capacitance, 25 μF; voltage 2.5 KV; pulse, 200 Ohms [Dower et al., 1988]). Immediately after 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 microcentrifuge and incubated at 37°C for 1 hr. A volume of 10 to 100 μl of the cells were spread on an agar plate containing the antibiotic to which the plasmid confers resistance.

RESULTS

PART I. GENOME SIZE OF *K. PNEUMONIAE* M5a1

A. Restriction enzyme used for genome mapping.

K. pneumoniae strains are known to have 56-58% G+C content (McClelland et al., 1987). Because the frequency of cutting depends both on the length of the recognition sequence and on the base composition of the chromosome, several rare cutting restriction endo-nucleases (octanuleotides and hexanucleotides) were screened to identify the enzymes appropriate for analysis of the *K. pneumoniae* chromosome. As expected the octanucleotide cutters, which recognize GC rich sequences, [*Sfi*I (GGCCNNNN/NGGCC), *Not*I (GC/GGCCGC), *Sgr*AI (C(A/G)/CCGG(T/C)G) and *Sse* 8387I (CCTGCA/GG)] cleaved the M5a1 DNA into too many fragments to clearly separated by pulsed field gel electrophoresis (Table 1). Other endonucleases containing exclusively AT residues in their recognition sites *Dra*I (TTT/AAA), *Pac*I (TTAAT/TAA), *Ase*I (AT/TAAT), *Ssp*I (AAT/ATT) and thought to make few cuts in chromosome with high G+C content also generated too many fragments for convenient analysis. The third group of enzymes tested included those which recognize sequences containing the rare tetranucleotide CTAG [*Xba*I, *Nhe*I, *Spe*I and *Bln*I (isoschizomer of *Avr*II)] (Table 1).

The smaller the number of fragments generated by the restriction enzymes, the easier it would be to construct a restriction map of the whole chromosome.

TABLE 1. Recognition sequences of different restriction enzymes and the maximum size of fragments generated from digestion of the M5a1 chromosome.

Restriction enzyme	Nucleotides in recognition site	Size of the largest M5a1 fragment (Kb)
Sfi I	GGCCNNNN/NGGCC	150
Not I	GC/GGCCGC	100
Sgr AI	C(A/G)/CCGG(T/C)G	100
Sse 83871	CCTGCA/GG	smear
Dra I	TTT/AAA	150
Pac I	TTAAT/TAA	250
Ase I	AT/TAAT	50
Ssp I	AAT/ATT	50
Xba I	T/CTAGA	760
Nhe I	G/CTAGT	less than 50
Spe I	A/CTAGT	450
Bln I	C/CTAGG	800

Thus *Xba*I, which produced 20 fragments and *Bln*I which produced 19 fragments, were selected to estimate the genome size and to make a macro-restriction map of M5a1.

B. Analysis of DNA fragments of the M5a1 chromosome and estimation of the total genome size.

Several electrophoretic runs performed under different conditions were necessary to unambiguously separate the DNA fragments in bands that were suspected of being triplets or doublets. The fragments generated by *Bln*I and *Xba*I were designated B_1 , B_2...B_{19} and X_1, X_2 ...X_{20}, respectively in order of their descending size.

1. Fragmentation of the genome by *Bln*I restriction enzyme

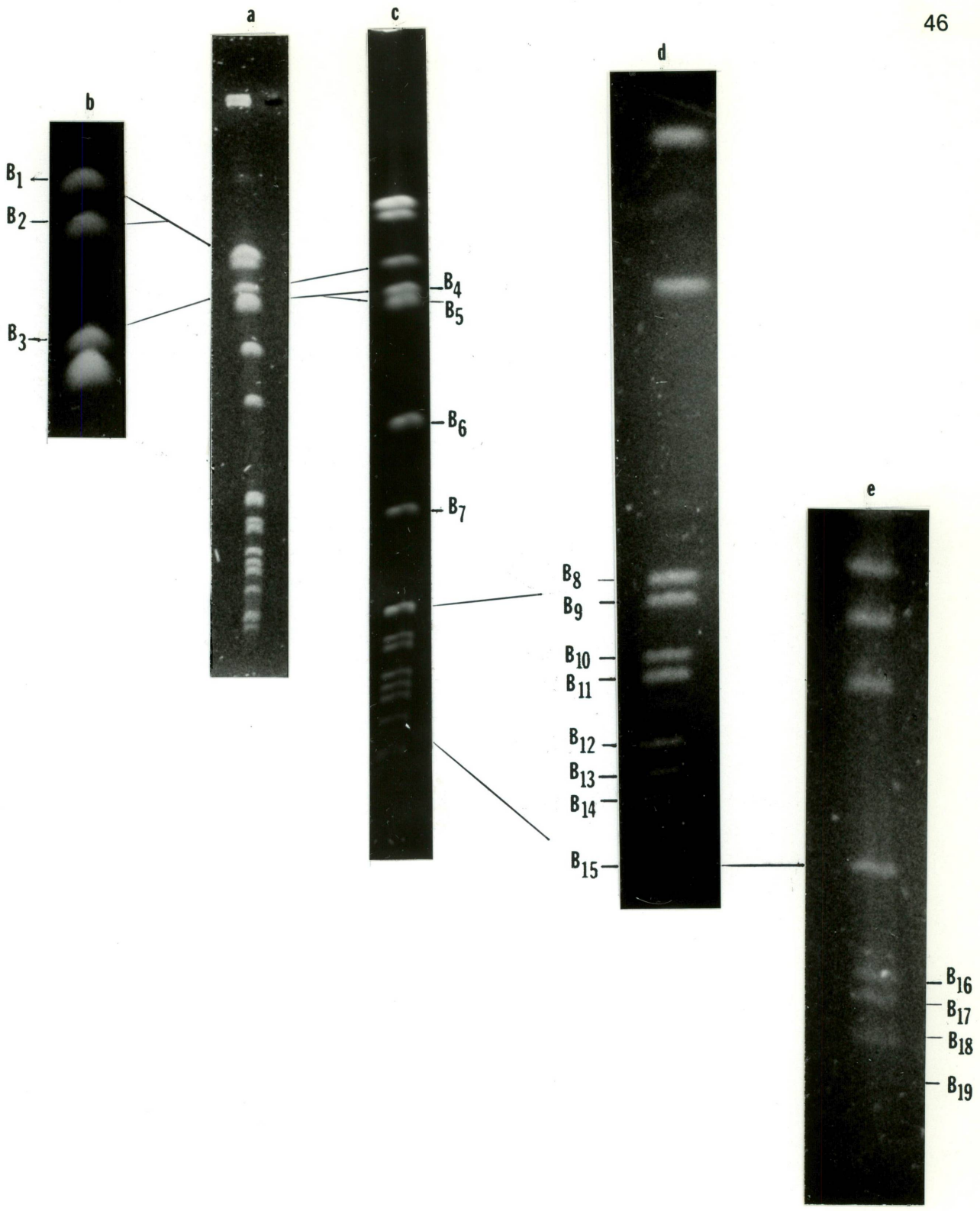
*Bln*I divides the M5a1 genome into 19 fragments which range in size from 805 Kb to 20 Kb (Table 2). A pulsed field electrophoretic run under standard conditions (25 sec for 16 hr, 45 sec for 10 hr and 100 sec for 14 hr), for example, can separate many of the *Bln*I fragments reasonably well and gives an over-view of the genomic restriction pattern for fragments larger than 100 Kb (Fig. 4a).

The fragments shown in Fig. 4a do not exhibit a monotonic reduction in ethidium fluorescence intensity as the fragment sizes decrease. Because of this it was suspected that several of the bands were composed of several superimposed fragments. High molecular weight B_1 and B_2 fragments were, therefore, further resolved using longer pulse times (Fig. 4b).

TABLE 2. Average fragment sizes of the M5a1 genome when digested by the restriction endonucleases *Xba*I and *Bln*I.

Digestion With *Xba*I		Digestion With *Bln*I	
Fragment designation	Kb	Fragment designation	Kb
X1	766	B1	805
X2	735	B2	740
X3	610	B3	576
X4	459	B4	555
X5	419	B5	531
X6	405	B6	420
X7	286	B7	326
X8	239	B8	190
X9	226	B9	176
X10	213	B10	152
X11	177	B11	145
X12	146	B12	114
X13	123	B13	106
X14	97	B14	89
X15	50	B15	61
X16	43	B16	50
X17	35	B17	45
X18	30	B18	38
X19	25	B19	20
X20	19		
Total size	5103Kb		5139Kb

Figure 4. Digestion of genome DNA of M5a1 using *Bln*I and separation of the fragments by pulsed field gel electrophoresis. Electrophoresis was performed in 1.2% agarose, 0.5 x TBE at 9°C and 170 V. The gel was photographed after staining with ethidium bromide in 0.5 x TBE for 15-20 min and removing the background stain by washing in 0.5 x TBE. a) Pulse times: 25 sec for 16 hr, 45 sec for 10 hr, 100 sec for 14 hr. b) Pulse times: 45 sec for 20 hr, 100 sec for 22 hr. c) Pulse times: 45 sec for 20 hr, 80 sec for 20 hr. d) The gel concentration was 1.5% and voltage 220 V. The pulse times 25 sec for 20 hr and 45 sec for 20 hr. e) The gel concentration was 1.8% and voltage 220 V. The pulse times were 5 sec for 15 hr, and 1 sec for 12 hr.


a
b
c
d
e
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
B16
B17
B18
B19

Similarly, a run using a pulse time targeting the 600 to 500 Kb range allowed the separation of B_4 and B_5 (Fig. 4c). Smaller fragments B_8, B_9, B_{10} and B_{11} were separated when the electrophoretic conditions targeted the 200 to 150 Kb range (Fig. 4d). The low molecular weight fragments of less than 100 Kb were resolved by applying two different short pulses and a higher agarose concentration (Fig. 4e).

2. Fragmentation of the genome by *Xba*I restriction enzyme

The chromosomal DNA of M5a1 was also digested with the *Xba*I enzyne. An overview of the genomic pattern obtained from a run under standard conditions is shown (Fig. 5a).

The largest fragments, X_1 and X_2, formed a doublet that were resolved into theirs component fragments at longer pulse times (Fig. 5b). Similarly Fig. 5c shows the separation of X_4, X_5, X_6, and Fig. 5d shows the separation of the triplet of X_8, X_9 and X_{10}, into bands of 239, 226, and 213 Kb, respectively. The fragments smaller than 100 Kb are shown resolved in Fig. 5e after applying shorter pulse times. The digestion of M5a1 with *Xba*I yielded a total of 20 fragments ranging from 760 Kb to 19 Kb (Table 2).

3. Determination of fragments sizes

The sizes of the *Xba*I and *Bln*I restriction fragments were estimated by comparing their migration with that of standard DNA molecular weight markers run on the same gel: the 5 Kb ladder (5-100 Kb range), lambda concatamers (50-1000 Kb range) and yeast chromosomes (225-1900 Kb range). Standard curves for the marker lanes were created for each gel by plotting the logarithm of the fragment

TABLE 2. Average fragment sizes of the M5a1 genome when digested by the restriction endonucleases *Xba*I and *Bln*I.

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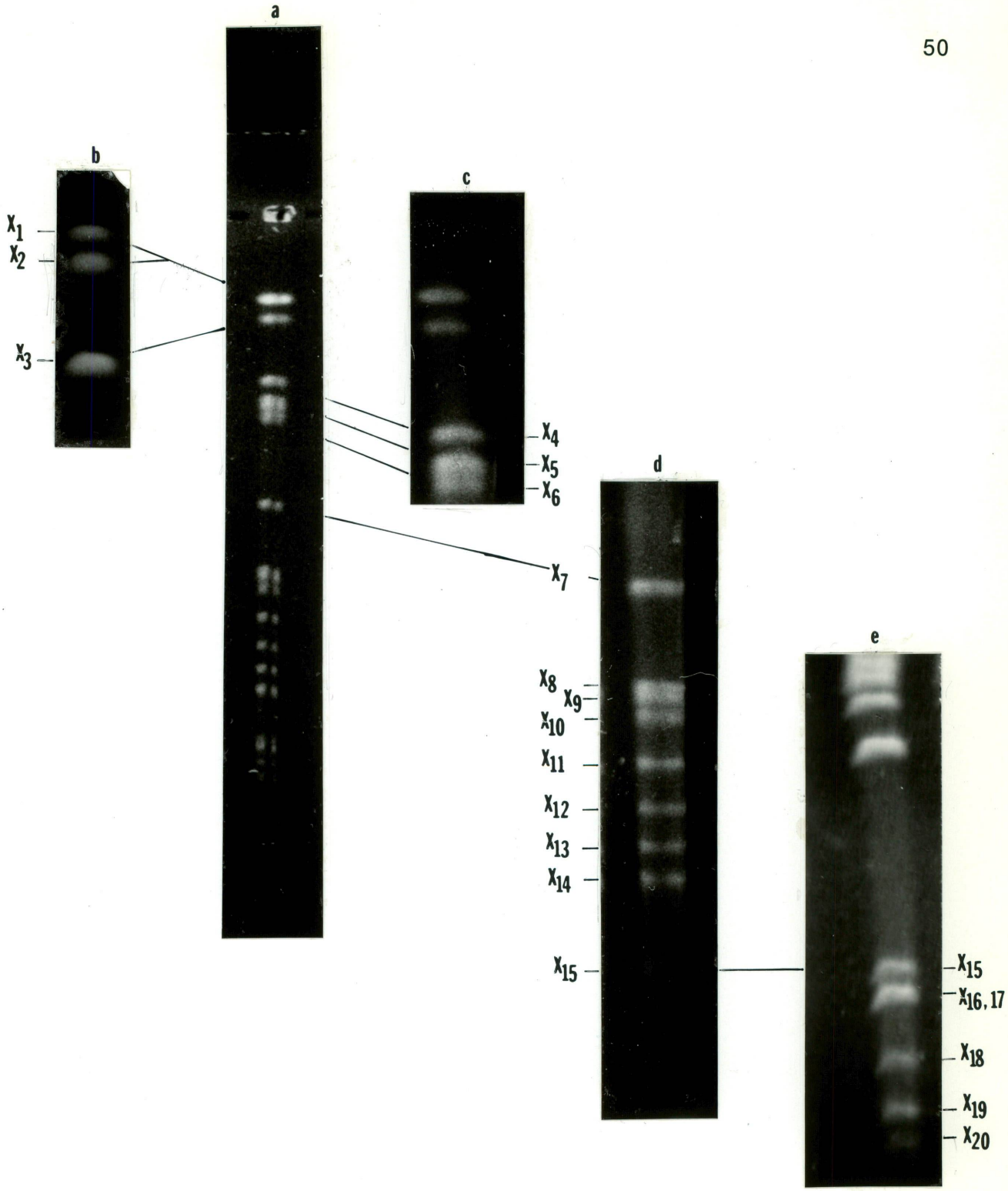
X5	419	B5	531
X6	405	B6	420
X7	286	B7	326
X8	239	B8	190
X9	226	B9	176
X10	213	B10	152
X11	177	B11	145
X12	146	B12	114
X13	123	B13	106
X14	97	B14	89
X15	50	B15	61
X16	43	B16	50
X17	35	B17	45
X18	30	B18	38
X19	25	B19	20
X20	19		
Total size	5103Kb		5139Kb

Figure 5. Digestion of genome DNA of M5a1 using *Xba*I and separation of the fragments by pulsed field gel electrophoresis. Electrophoresis was performed in 1.2% agarose, 0.5 x TBE, at 9°C and 170 V. The gel was photographed after staining with ethidium bromide in 0.5 x TBE for 15-20 min and removing the background stain by washing in 0.5 x TBE. a) Pulse times: 25 sec for 16 hr, 45 sec for 10 hr, 100 sec for 14 hr. b) Pulse times: 45 sec for 20 hr, 100 sec for 20 hr. c) Pulse time: 45 sec for 25 hr, 25 sec for 15 hr. d) The gel concentration was 1.5% and voltage 220 V. The pulse times were 25 sec for 10 hr and 15 sec for 17 hr. e) The gel concentration was 1.8% and voltage was 220 V. The pulse times 5 sec for 18 hr, and 1 sec for 12 hr.


a
b
c
d
e
X1
X2
X3
X4
X5
X6
X7
X8
X9
X10
X11
X12
X13
X14
X15
X15
X16, 17
X18
X19
X20

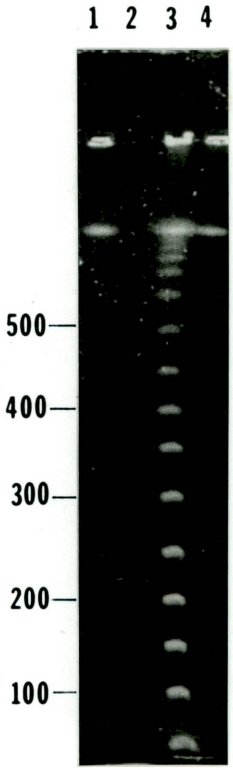
length in kilobases versus the distance in millimeters traveled from the wells. The distances migrated by each M5a1 DNA fragment were then used to estimate fragment size by interpolation from the standard curves. The size of each fragment was determined in several runs and the mean of the values was used. The total genome size was estimated by summation of the individual mean fragment lengths. From the *Xba*I digests the sum was 5103 Kb (± 2.7% sd) while the *Bln*I digests gave a total of 5139 Kb (± 2.9% sd) (Table 2). From these two values an estimate of 5121 Kb (±2.8% sd) was obtained for the size of M5a1 genome.

Fragments smaller than 20 Kb may not have been detected in these gels because they diffuse more rapidly and because the intrinsic intensity of their fluorescence after ethidium bromide staining is low.

To check the possibility of having large plasmids in M5a1, undigested chromosomal DNA was run on PFGE. Most of the bacterial DNA remained in the starting well, with some undigested DNA migrating into the gel during the electrophoresis (Fig.6). DNA from this strain was also digested with S1 nuclease and subjected to PFGE, no plasmid DNA was detected (Bret Barton, personal communication). This illustrates that M5a1 probably has no large plasmid. Further, this experiment suggests that the chromosome is circular, because of the failure of the vast majority of the undigested chromosomal DNA to migrate under pulsed field gel electrophoresis (Krause and Mawn, 1990; Alleman et al., 1993).

The possible presence of plasmids of less than 20 kb in M5a1 was also checked by Valinluck (1992). A regular electrophoresis gel was run using an uncut

Figure 6. Pulsed field gel electrophoresis of undigested M5a1 genomic DNA completed in 0.5 x TBE, 170 V at 9°C, with an agarose gel concentration of 1.2%. The gel was photographed after staining with ethidium bromide in 0.5 x TBE. The gel run at 25 sec for 15 hr, 45 sec for 25 hr and 100 sec for 2 hr. Lanes 1 and 4 M5a1 undigested chromosomal DNA, lane 2 unused, lane 3 lambda ladder.


1 2 3 4
500
400
300
200
100

chromosomal DNA sample. No plasmids were detected, thus suggested that M5a1 has no plasmids.

For a comparison, the genome size of another strain of *K. pneumoniae* GM236, was also digested with the two same rare cutting restriction enzymes (*Xba*I and *Bln*I). This strain contains a different restriction modification system, KpnBI (Valinluck, 1992). This analysis produced a preliminary estimate of 4763 Kb (based on *Xba*I digest) and 4838 Kb (based on *Bln*I digest) as the total genomic size GM236.

Part II. Construction of a physical map of the *K. pneumoniae* M5a1 genome

A. Orientation of the chromosomal restriction fragments by cross-hybridization.

Determination of the relative order of the restriction fragments of M5a1 was accomplished by two method based on Southern hybridization. The first method used restriction fragments as probes and the second method used cloned DNA as probes. The principle behind the Southern analysis of pulsed field gel electrophoresis is the same as the conventional analysis used to characterize regular electrophoresis gel. However, in the case of PFGE gel, the efficiency of transfer of DNA is less due to the largeness of the fragment sizes. This results in less detectable hybridization and in diffuse bands.

In the first approach, chromosomal DNA of M5a1 was digested with one of the two restriction enzymes *Xba*I and *Bln*I and the fragments were separated using PFGE. Each fragment was then labelled with ^{32}P (dCTP). The labelled fragments

were used as probes in Southern hybridization experiments with M5a1 chromosomal DNA cut with the other restriction enzyme and separated by PFGE. If one fragment hybridized to two or more fragments of another digest, we used this as evidence for an overlap between those fragments. For example, fragment B_9 was found to hybridize to fragments X_3 and X_{11} (Fig. 7). Similarly, fragment X_4 was found to hybridize to B_1, B_6 and B_{15} (Fig. 8).

All *Bln*I and *Xba*I fragments were excised and purified from the agarose gels. Most fragments were resolved as single bands but fragments X_4, X_5, X_6, and X_8, X_9, X_{10}, which did not resolve well, were cut out as multiplets. This reciprocal hybridization allowed me to determine the overlap and orientation of corresponding fragments from different restriction digests. The final form is shown as a circular physical map (Fig. 9).

The map, however, still presented an ordering problem when two or three fragments hybridized to the same fragments, for example X_{14}, X_{12}, and X_{15} to fragment B_3, and B_{11} and B_{18} hybridized to the fragment X_{10}. To determine their orders a second set of Southern hybridization experiments was performed. In these experiments, known genetic clones were used as probes and the chromosomal DNA was partially digested. For this purpose random clones and clones complementing auxotrophic requirements were obtained from our laboratory.

To obtain a clone which can complement a specific nutritional requirement, a chromosomal DNA library of M5a1 was constructed using a plasmid pBR322.

Figure 7. Southern hybridization between fragments B_9 (probe) and X_3 and X_{11}. B_9 represents a linking fragment. Panel A shows the portion of the map constructed based on this hybridization. The outside circle represents the *Xba*I digest, and the inside circle represents the *Bln*I digest. Panel B shows the autoradiography result of the hybridization used to construct that portion of panel A.

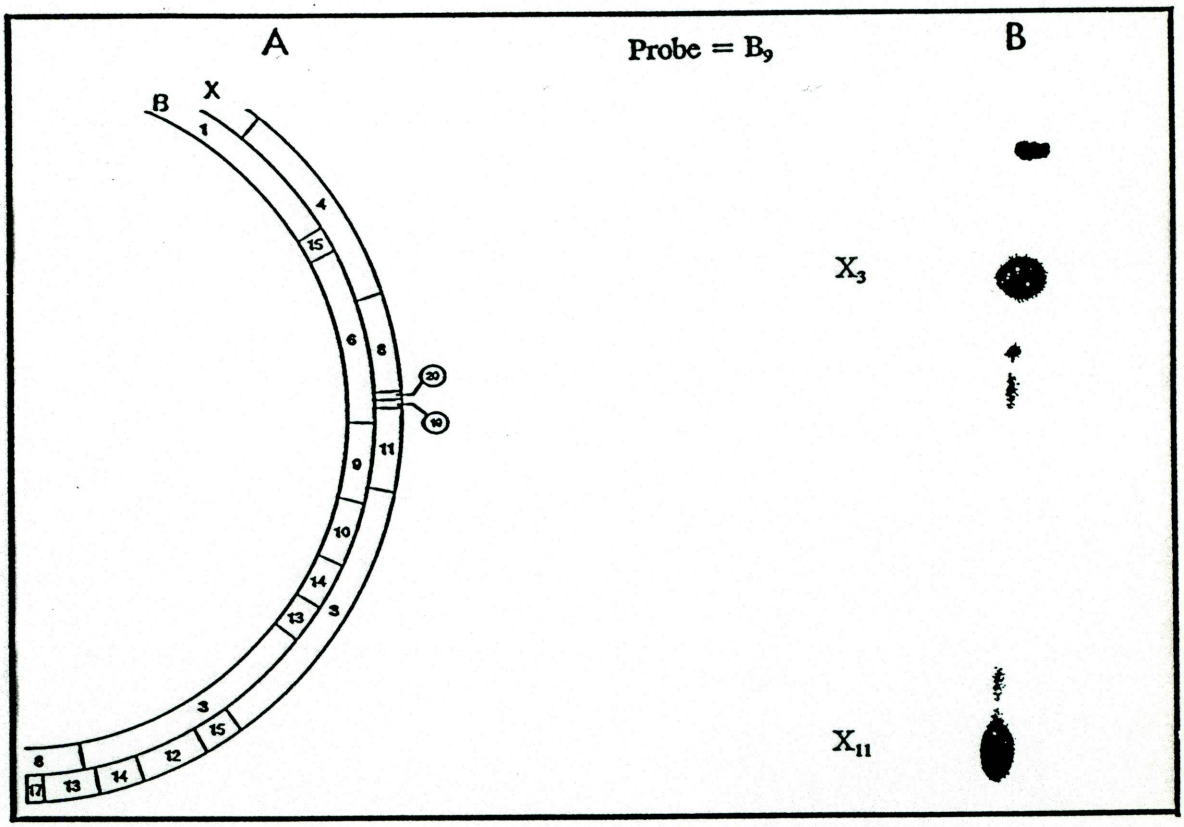

A
Probe = B_9
B
B
X
1
4
15
6
8
20
10
9
11
10
14
13
3
3
15
8
12
17
13
14
X_3
X_{11}

Figure 8. Southern hybridization between fragment X_4 (probe) and B_1, B_6, and B_{15}. Panel A shows the portion of the map constructed based on this hybridization. The outside circle represents the *Xba*I digest, and the inside circle represents the *Bln*I digest. Panel B shows the autoradiography result of the hybridization used to construct that portion of panel A.

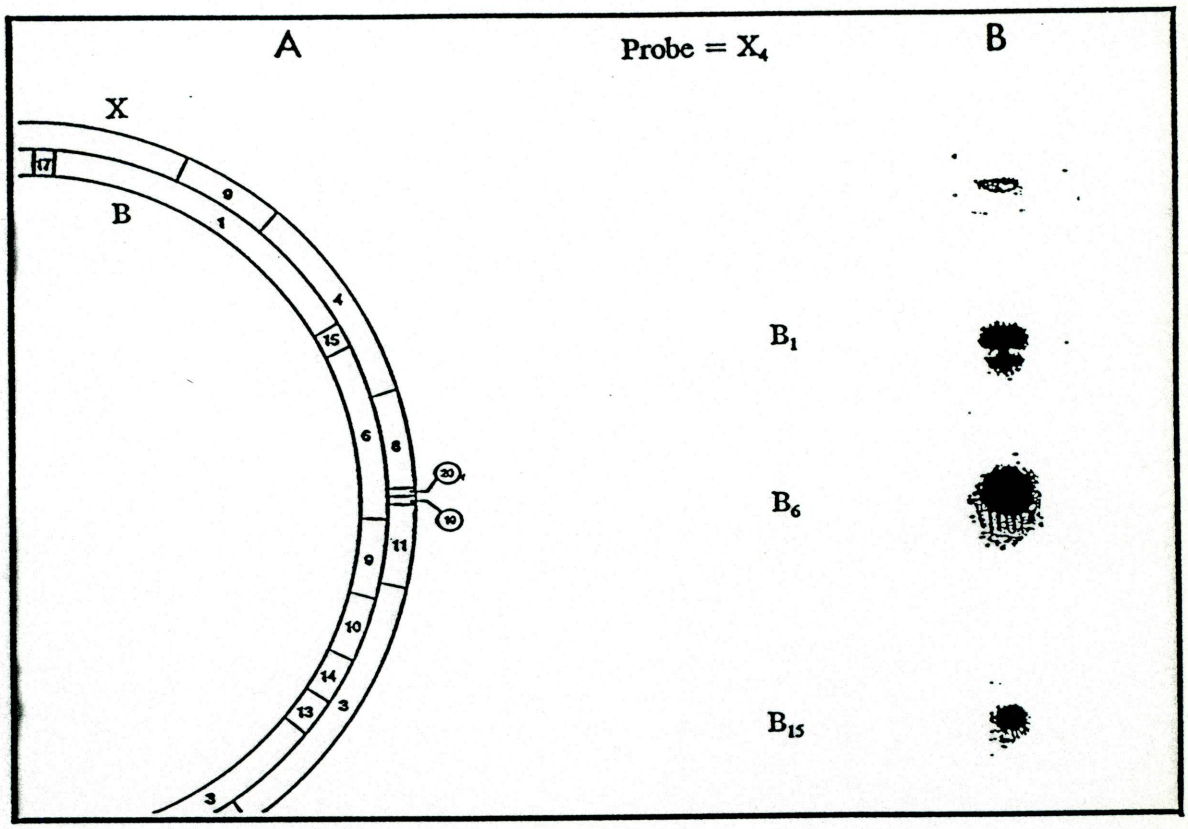
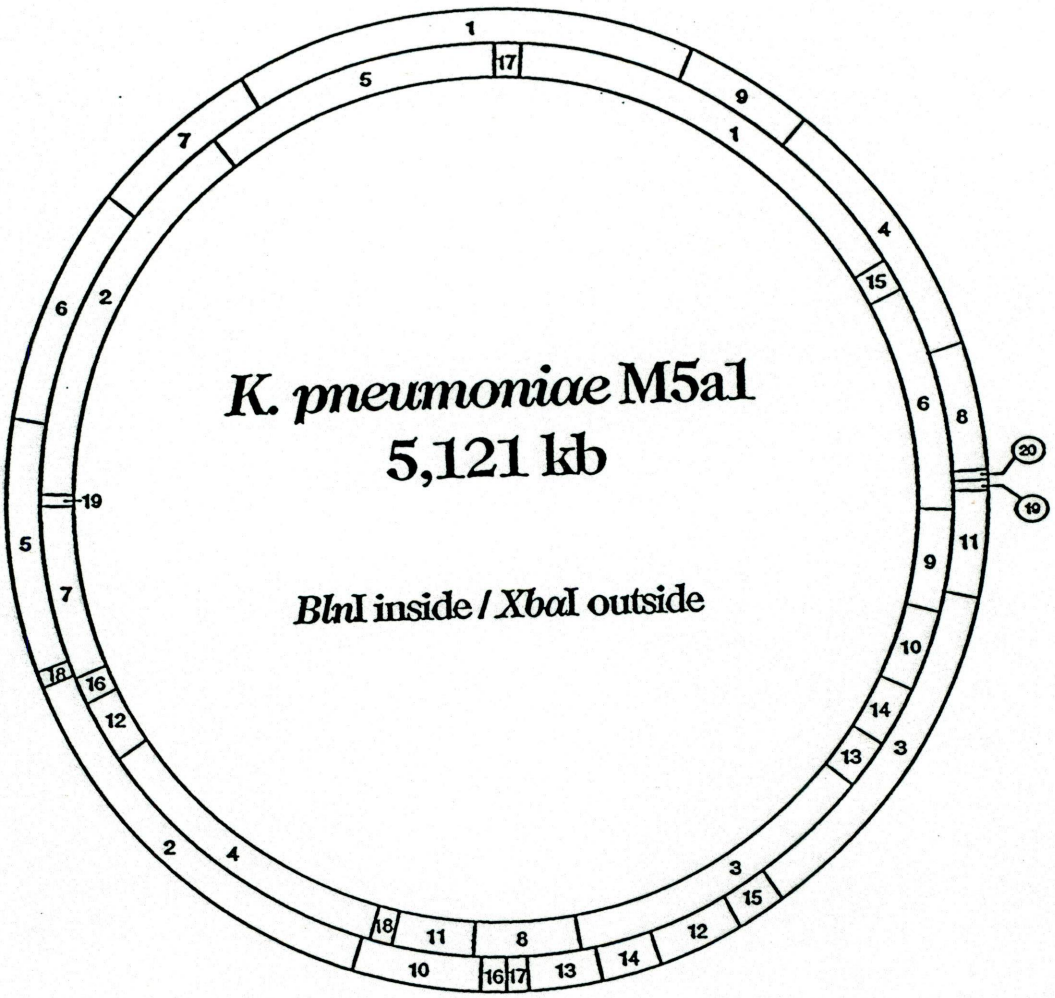

A
Probe = X4
B
X
17
B
9
1
4
15
6
8
20
10
11
0
10
14
13
3
3
B1
B6
B15

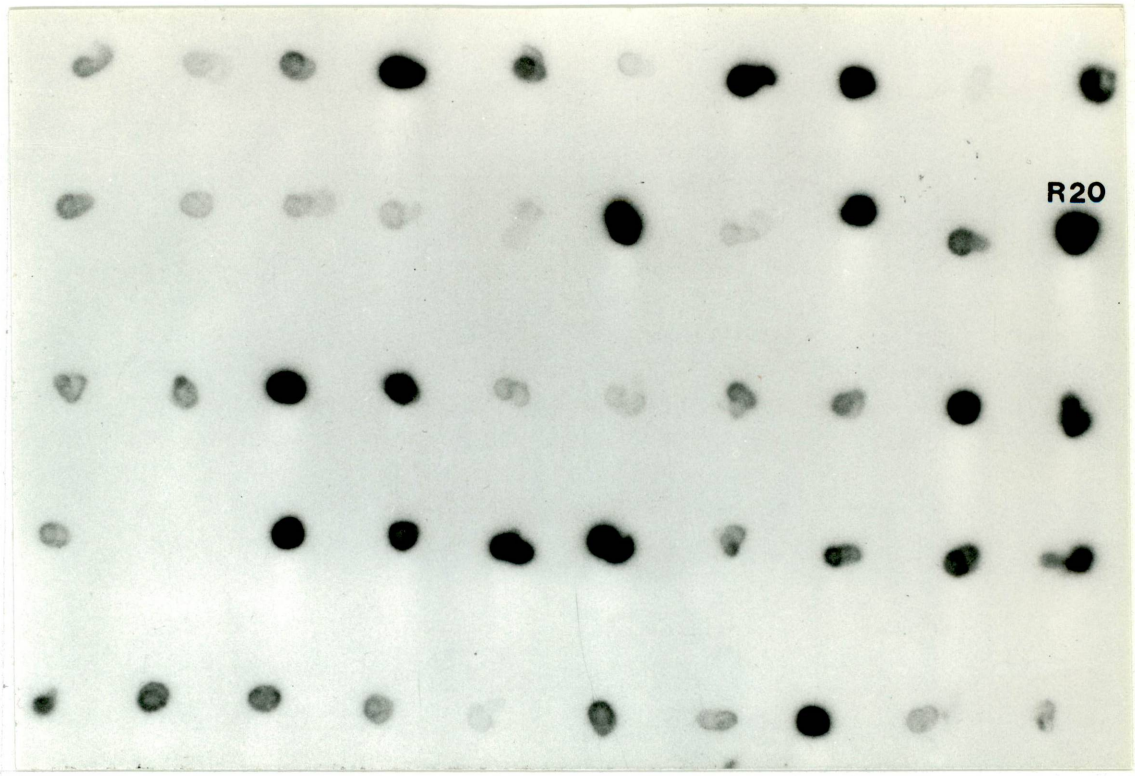
Figure 9. A physical map of the M5a1 genome. The outside circle represents the *Xba*I digest, and the inside circle represents the *Bln*I digest. Fragments are numbered from the largest size to the smallest.

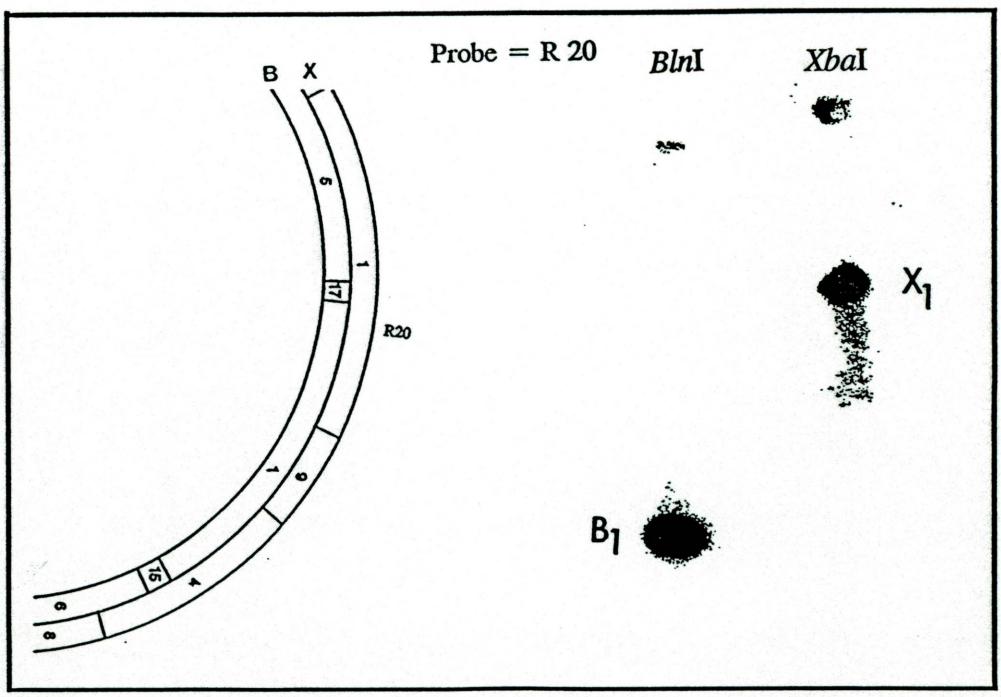

K. pneumoniae M5a1
5,121 kb
BlnI inside / XbaI outside

M5a1 DNA was partially digested with *Sau*3AI and ligated to *Bam*HI digested and dephosphorylated pBR322 . The ligated plasmid DNA was first transformed and amplified in *E. coli* XL-1 Blue. The DNA from these random chromosomal DNA library clones was spotted onto a nylon membrane. In an alternate approach, chromosomal DNA of M5a1 was digested with either *Bln*I or *Xba*I,then purified and radioactively labelled with ^{32}P. These probes were used to hybridize the random clone described above. Clones which hybridized strongly to a specific fragment were selected and then used as probes against membranes of the *Xba*I or *Bln*I digested M5a1 chromosomal DNA. This reciprocal hybridization technique allowed me to confirm some of the connections of the *Bln*I and *Xba*I fragments in Fig. 9. An illustration of this method of mapping can be seen in Fig. 10. Random chromosomal DNA library clones were dot blotted onto a nylon membrane and probed with a ^{32}P-labelled X1 fragment (Fig. 10A). As seen in Fig. 10, a random clone R 20 showed strong hybridization to the probe and was chosen to reciprocally hybridize to the digested DNA which was separated by PFGE. Figure 10 B shows the confirmation of the hybridization between R 20 and both B_1 and X_1 using Southern hybridization. This procedure also fortuitously yielded a clone R24 which made it it possible for me to order X_{15} and X_{12}. When R24 was used as a probe against *Xba*I partially digested M5a1 chromosomal DNA, the hybridization result showed that X_{15} and X_{12} form contigs (connected fragments). As for fragment X_{14}, its location could not be definitively assigned by these data. Therefore X_{14} could be on either side of the X_{12}-X_{15} contig, i.e X_{14} can be either next to X_3 or X_{13}. The same library

Figure 10. Southern hybridization between chromosomal fragments and random clones. A. A nylon membrane showing a grid of random clone DNA spots. The membrane was probed with fragment X_1. Random clone R_{20} was selected for reciprocal hybridization. B. Southern hybridization between R_{20} (probe) and X_1 and B_1.

Probe = X_1



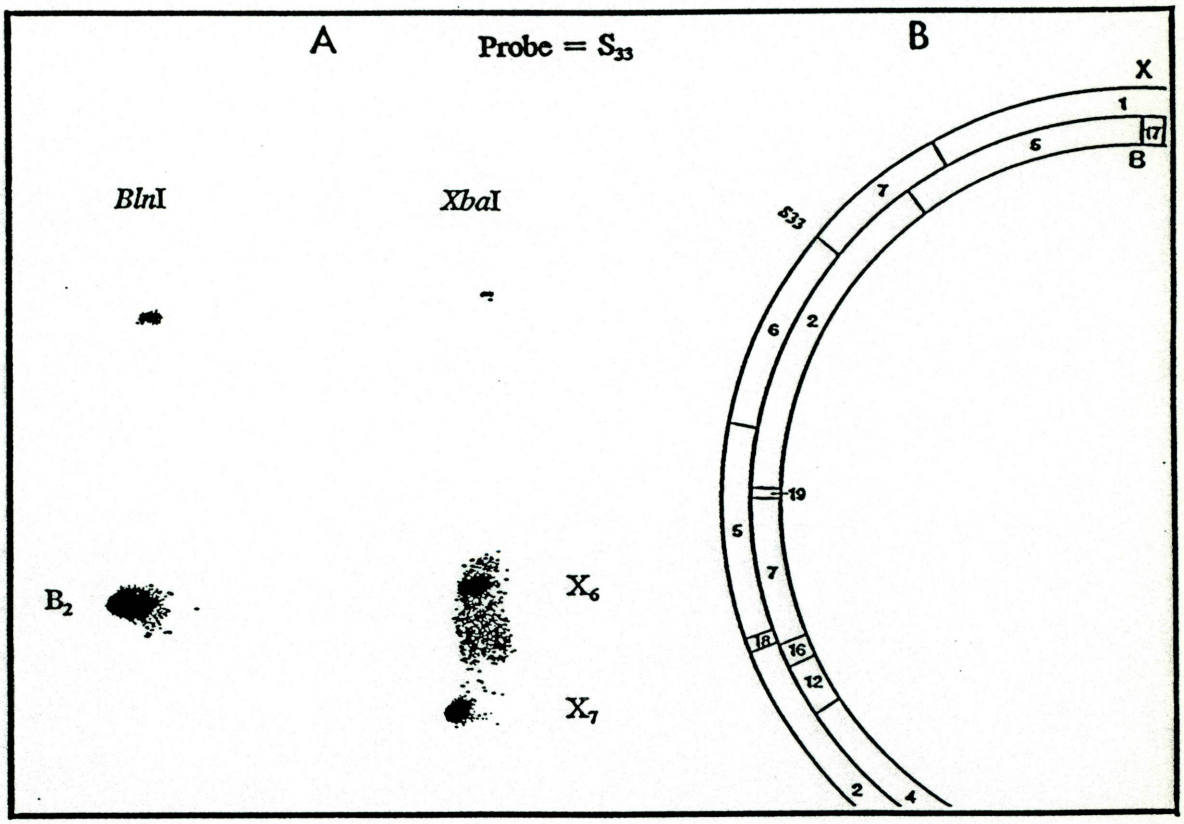


generated a so-called "linking clone", S33. This clone was found to hybridize to two fragments of *Xba*I digests, X_6 and X_7, and to only one fragment of *Bln*I digest, B_2 (Fig. 11). This linking clone confirmed the map established in Fig. 9. Similarly, the *phoE* gene seemed to be also a linking clone and found to hybridize to two fragments of the *Bln*I digests, B_{10} and B_{14} and to one fragment of a *Xba*I digests X_3, allowing the determination of their relative order.

The restriction map in Fig. 9 still contains some ambiguities. Fragments X_{16} and X_{17} both hybridized to fragments B_8, and X_{19} and X_{20} both hybridized to fragment B_6. Their positions relative to each other were therefore assigned arbitrarily. Similarly, B_{11} and B_{18} hybridized to the same fragment, $X_{10,}$ and could not be precisely ordered within the physical map. The positions of fragments X_{19} and X_{20} may be reversed or both of these fragments may be located on the other side of X_8 next to X_4. Additionally, X_{19} and X_{20} may not be connected but may be located on opposite sides of X_8 . Fragments B_{18} and B_{11} may be located in the reverse position.

Having completed a map for the entire genome of M5a1, the next step was to assign a site for the 0 minute on the map. For this purpose, *thr*$^+$ clone was isolated from the random chromosomal library obtained above. The same chromosomal DNA library was first amplified in *E. coli* XL-1 Blue and then transformed to *thr*$^-$ auxotrophic mutants of M5a1 developed in our laboratory. A clone identified by complementing a threonine auxotrophic mutation was obtained, the clone was designated pAJ1 and the fine restriction mapping of the

Figure 11. Southern hybridization between a random clone S_{33} (probe) and X_6, X_7 and B_2 fragments. On the *Xba*I digest, S_{33} represents a linking clone building to the adjacent fragments X_6 and X_7. Panel A shows the autoradiography result of the hybridization. Panel B shows the portion of the map constructed based on this hybridization.


A
Probe = S33
B
X
1
17
5
B
7
533
6
2
19
5
7
18
16
12
2
4
BlnI
XbaI
B2
X6
X7

clone was constructed using *BamH*I, *Hind*III and *Eco*RI (Fig. 12). The threonine gene was found clearly to hybridize to fragments B_1 and X_1 (Fig. 13).

B. Gene mapping of other *K. pneumoniae* clones

As an expansion of my thesis research I mapped several clones obtained from various laboratories in the world which work with strains of *K. pneumoniae* (Table 3). These clones were used as probes for Southern hybridization against M5a1 chromosomal DNA membranes. Of all the genes acquired from other laboratories only the *pulA* clone showed no hybridization to the M5a1 genome. This unexpected result may have occured because the M5a1 strain cannot use pullulan as a carbon source. Figure 14 shows that a clone carrying the *nifJ,HDK* gene (the nitrogenase structural protein), hybridized to X_3 and to B_3. These hybridization results further confirm the constructed physical map. Accordingly, a comprehensive physical map which includes the localized genes was made (Fig. 15). The relative order and location of the genes in the same overlapping fragment, for example, *nifJ,HDK,* type 3 *fimbriae* and *nac* in the X_3 and B_3 overlapping region have not been determined.

Figure 12. Restriction map of clone pAJ1, a clone which complemented *thr* requiring auxotrophic mutant. The insertion is 2.7 Kb. The vector pBR322 is shown by thick shadow while the insert is shown as a double line. Restriction endonuclease site is indicated.

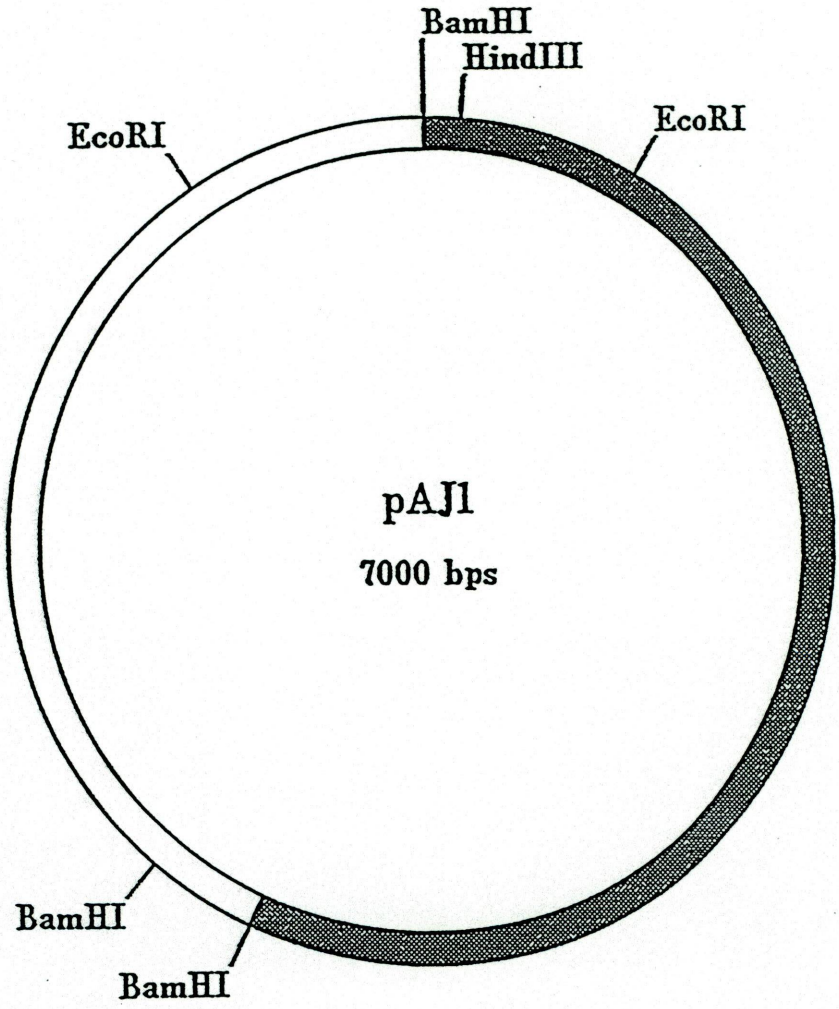

BamHI
HindIII
EcoRI
EcoRI
pAJ1
7000 bps
BamHI
BamHI

Figure 13. Southern hybridization between the *thr* clone and X_1 and B_1. This result defined the zero minute on the map. Panel A shows the portion of the map constructed based on this hybridization. The outside circle represents the *Xba*I digest, and the inside circle represents the *Bln*I digest. Panel B shows the autoradiography result of the hybridization used to construct that portion of panel A.

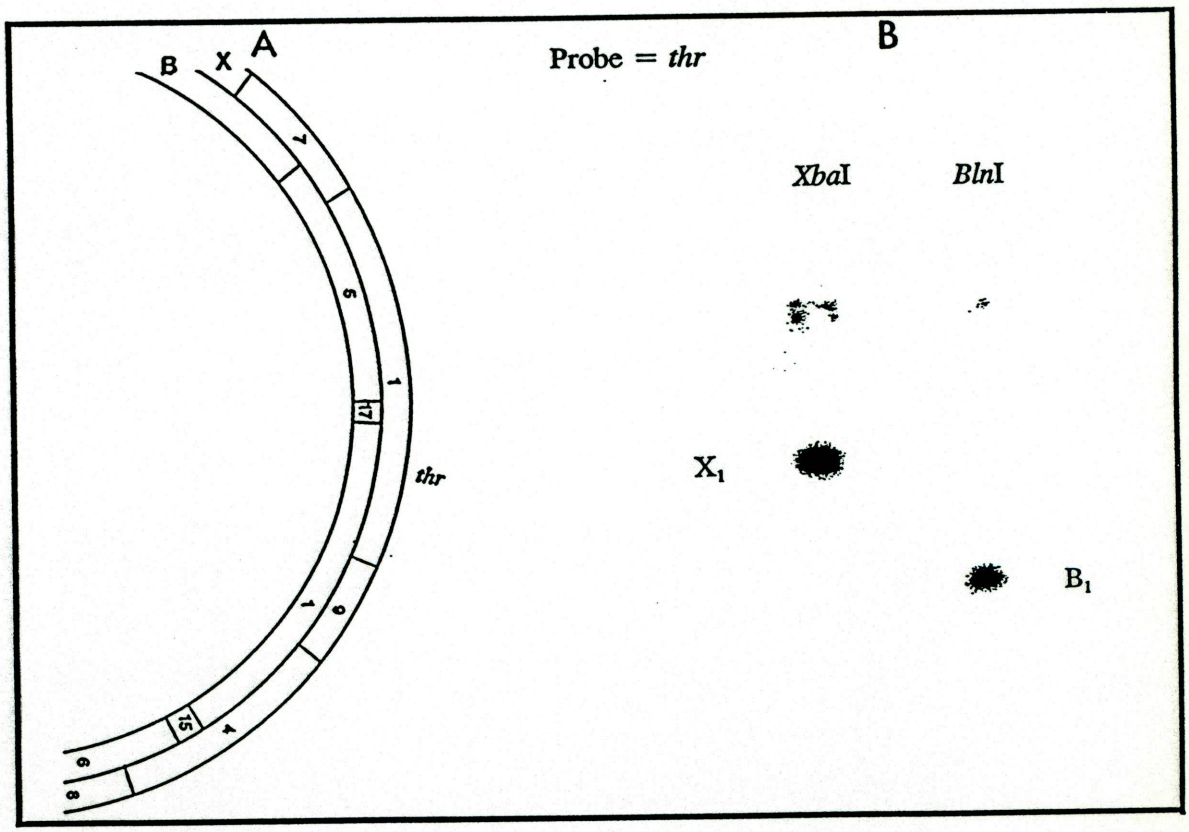

A
B
X
thr
B
Probe = thr
XbaI
BlnI
X1
B1

TABLE 3. *K. pneumoniae* genes and plasmids

Gene Cloned	Plasmid	Sources
Lab. clones		
KpnA	pUC18	Ryu
pur	pBR322	This study
thr	pBR322	This study
ilv5	pBR322	This study
pro	pBR322	This study
ura1	pBR322	This study
ura2	pBR322	This study
Random clones		
R20	pUC18	This study
R24	pUC18	This study
R30	pUC18	This study
R45	pUC18	This study
S33	pUC18	This study
S47	pUC18	This study
Clones obtained from others labs		
glnA, glnL, glnG	pGE100	M. Merrick. Univ. of Sussex England
rpoN	pMM17	Same as above
β-galactosidase	pCR100	M. Riley-Marine Biol. Lab Massachusetts
glnA, glnL,	pACR34	A. Covarrubias-Univ. Nacional autonoma de Mexico
β-lac, ampC, fum.red.	pNU5	S.Bergstrom-Univ. of Unea Sweden
urease operon	pKAUI17	R.P. Hausinger-Michigan State Univ.
Type 3 fimbrae	pFK10	S. Clegg- University of Iowa
narXL	pVJS429	V. Stewart- Corvell Univ.

TABLE 3. *K. pneumoniae* genes and plasmids (continued)

Gene Cloned	Plasmid	Sources
hut	pCB101	R.A. Bender - Univ. of Michigan
nac	pCJ5	Same as above
crp	pJP13	Same as above
gdhA	pGDH4	Same as above
phoE	pKP2	J. Tommassen- Univ. ofUtrecht/Netherlands
rfb	pWQI	C. Whtifield - Univ. of Guelph/Canada
rcsA	pWQ100	Same as above
nifJ,HDK	pUC362	G. Roberts- Univ. of Winsconsin
pul A	pCHAP200	A.P. Pusgley- Institut Pasteur/France

Figure 14. Southern hybridization between the *nifJ, HDK* clone (probe) and X_3 and B_3. Panel A shows the portion of the map constructed based on this hybridization. The outside circle represents the *Xba*I digest, and the inside circle represents the *Bln*I digest. Panel B shows the autoradiography result of the hybridization used to construct that portion of panel A.

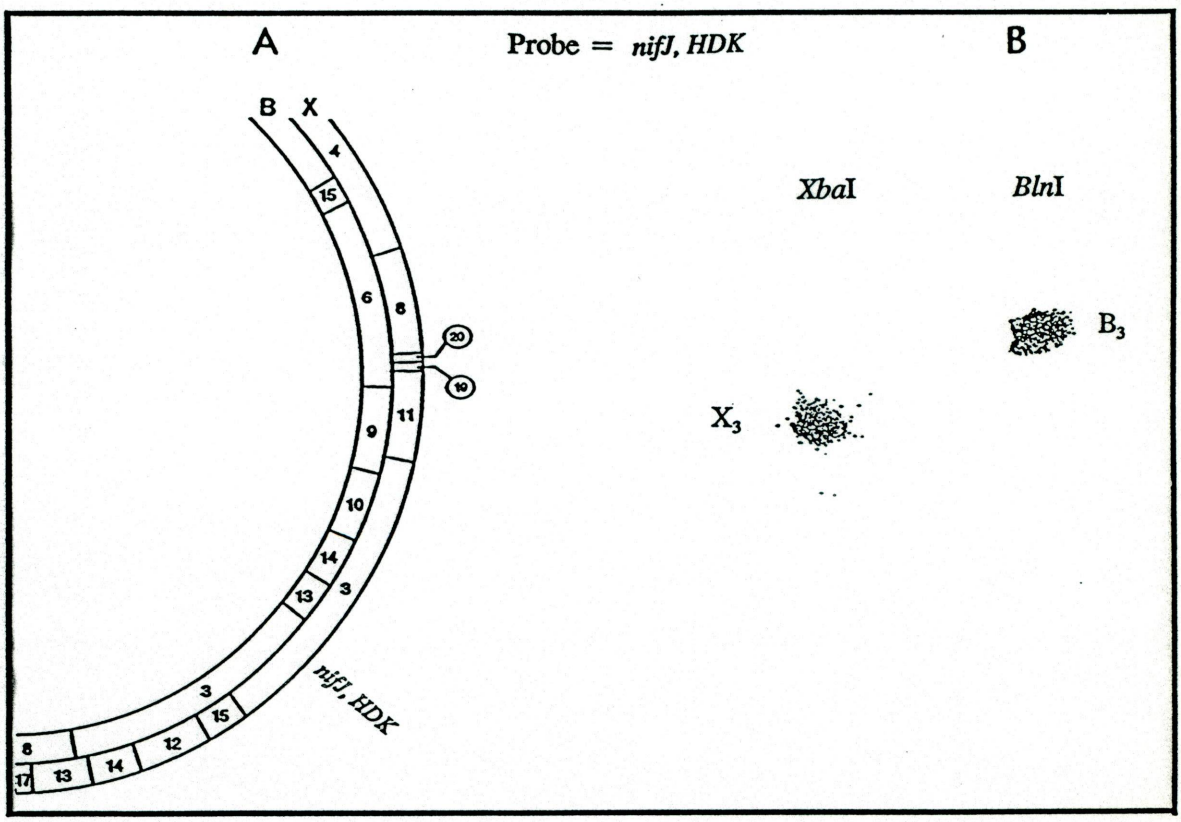
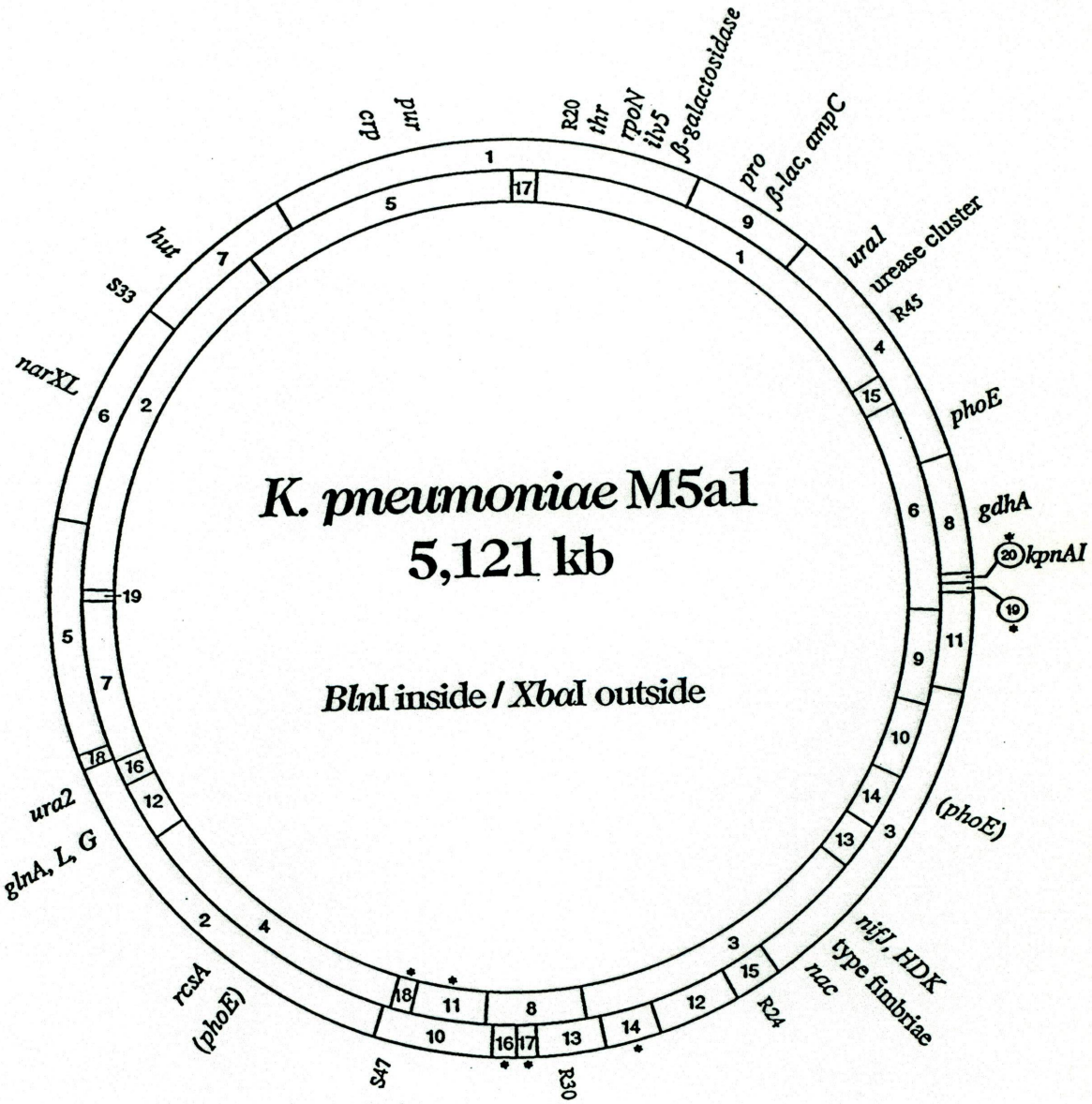

A
Probe = nifJ, HDK
B
B X
4
15
6
8
20
19
11
9
10
14
13
3
3
15
12
14
13
17
8
nifJ, HDK
XbaI
BlnI
B3
X3

Figure 15. A physical map of the M5a1 circular chromosome with mapped genes. The genes can be reliably placed within the fragments noted, but there relative orders and locations within the fragments are not known. *phoE* indicates the expected hybridization region for the clone. *phoE* in parentheses indicates other definite hybridization to the same probe. Ambiguous order of fragments is marked by asterisk.

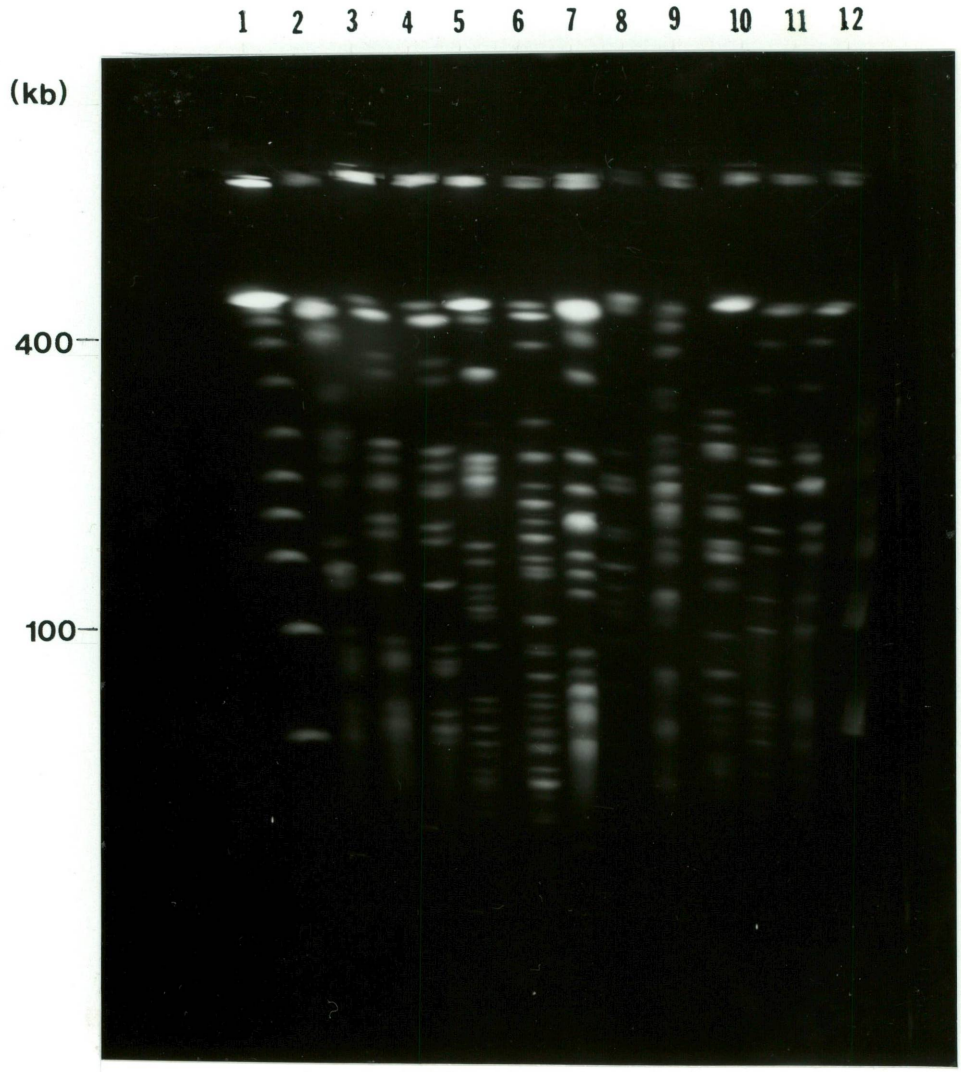

K. pneumoniae M5a1
5,121 kb
BlnI inside / XbaI outside
crp
pnt
R20
thr
rpoN
ilv5
β-galactosidase
pro
β-lac, ampC
ura1
urease cluster
R45
phoE
gdhA
kpnAI
(phoE)
nifJ, HDK
type fimbriae
nac
R24
R30
S47
rcsA
(phoE)
ura2
glnA, L, G
narXL
S33
hut

Part III. DNA Fingerprinting of clinical samples.

DNA fingerprinting is a process of strain identification which has many applications. In a hospital a definite means of identification may be of importance in some cases. I tested the potential use of this method with *K. pneumoniae* isolated from hospital samples. The patient samples were collected from different locations in the body, including patient urine, sputum, nasal drain and peritoneal cavity. About 50 samples were collected during the years 1991-1992 at the Loma Linda University Medical Center, cultured at the hospital clinical microbiology laboratories and passed on to me. In addition, other clinical samples were obtained from the laboratory of Dr. L. Bullas. These samples were also originally collected at the LLMC during the year 1981. In this study I examined patterns of restriction fragments of the chromosomal DNA of the various clinical samples. The DNA from clinical samples was cut with *Xba*I and subjected to PFGE. The pulsed field gel electrophoresis was run under conditions targeting small fragments, of less than 300 Kb. Figure 16 shows a typical fingerprint patterns obtained with the samples.

An overview of the fingerprints shows a variety of fingerprinting patterns. In spite of the fact that the samples originated from different parts of the bodies of different patients, some show identical patterns. The identical

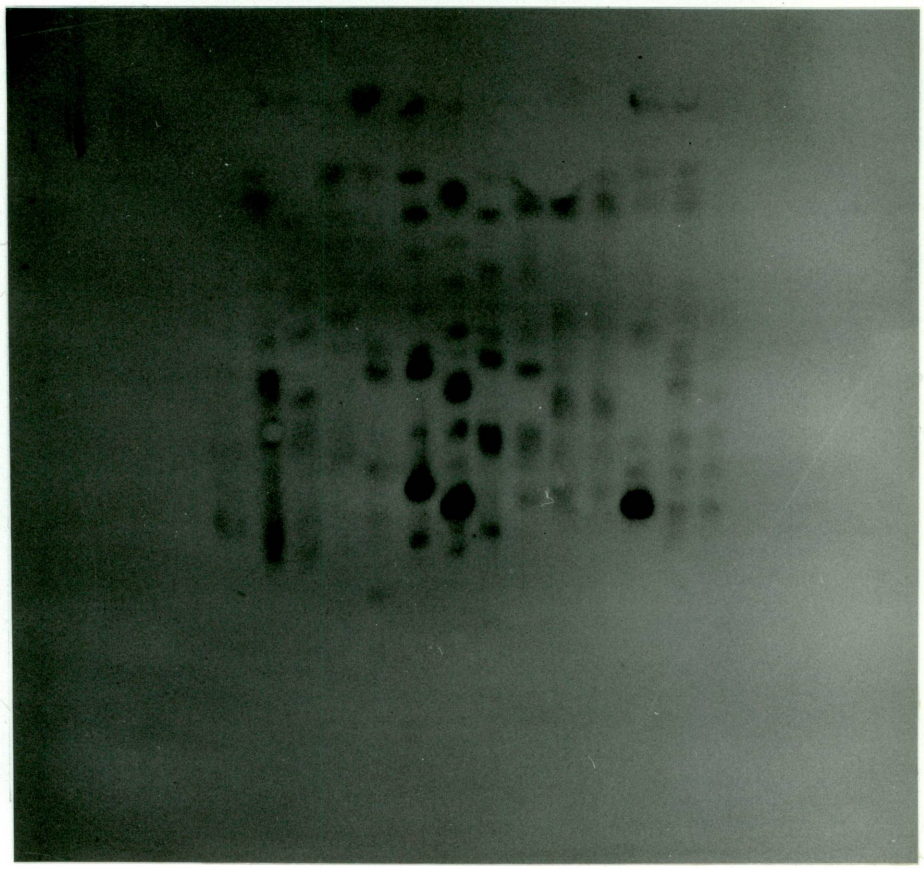
Figure 16. Clinical *K. pneumoniae* DNA samples cut with *Xba*I and separated by pulsed field gel electrophoresis. Note the diversity of patterns among the different isolates. Lanes 3 and 4, and lanes 11 and 12 show pairs of identical *Xba*I fingerprints respectively. Lane 1 lambda ladder; Lanes 2,3,4 and 5 are samples from urine; Lanes 6,7 and 8 are strains collected in 1981; Lane 9 sample from sputum; Lane 10 sample from urine; Lanes 11 and 12 are samples from peritoneal tissue.


1 2 3 4 5 6 7 8 9 10 11 12
(kb)
400
100

fingerprints were shared by two sets of samples from the same biological location, two bacterial isolates from the urine of different patients (lane 3 and lane 4) and two other isolates from the peritoneal cavity of yet two more patients (lane 11 and lane 12) within the sets. Further characterization of the fingerprints was made by hybridizing the *nif* genes to the chromosomal DNA of the clinical samples (Fig. 17). The hybridization showed that although nitrogen fixation is common among *K. pneumoniae* strains, the *nif* are not present in all *K. pneumoniae* clinical strains tested. This result indicates that the *nif* genes may potentially be used as systemic markers.

Figure 17. Chromosomal DNA from *K. pneumoniae* urine clinical samples from different patients probed with *nifJ,HDK* gene.

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



DISCUSSION

K. pneumoniae is an enteric bacterium commonly found in the normal gut flora. In this study, I characterized the genome using restriction enzymes and pulsed field gel electrophoresis. The rare cutting enzymes *Bln*I and *Xba*I were utilized throughout the study. This work led to the construction of the first physical map of a *K. pneumoniae* strain. I established that the *K. pneumoniae* M5a1 chromosome is circular and is 5121 Kb $\pm$ 2.8% in size. A second feature revealed by this work is the heterogeneity in electrophoretic patterns of the *Xba*I digest of different *K. pneumoniae* clinical isolates. The availability of the *K. pneumoniae* genome map will allow further studies of the chromosome at a more refined molecular level.

The genome size of *K. pneumoniae* was previously estimated to be 3,300 Kb to 4,600 Kb by renaturation kinetics (Herdman, 1985). This differs from the 5121 Kb size determined by pulsed field electrophoresis in this study. Similarly, for *E. coli* (3787 Kb) and for other prokaryotic organisms, the chromosome size estimated by renaturation kinetics was less than the estimate deduced from pulsed field gel electrophoresis (Hector and Johnson, 1990). This difference can be simply explained as a result of impurities in the DNA samples, or in an alternate view, if there are multiple copies of the same gene in the chromosome, then the renaturation kinetics can give erroneous results (Pyle et al., 1988, Alleman et al., 1993).

There is quite a range of variation in total genome size among bacterial species. There is a general trend that bacteria which are obligatory intracellular parasites and rely on their cellular hosts for the provision of many of their nutritional

requirements, tend to have small genomes (Appendix). For example, the genome of such obligate parasites as *Mycoplasma* (580 Kb to 1300 Kb), *Rickettsiella* (1700 Kb to 2000 Kb) and *Chlamydia* (1000 Kb to 1500 Kb) are small, while free-living forms tend to have larger genomes up to 9454 Kb for *Myxococcus scanthus*. Genome sizes are also often related to the morphological and physiological complexities of the organism. The difference of the size between the large genome (5826 Kb) of *Pseudomonas aeruginosa* PAO, 5862 Kb and that of *E. coli* (4700 Kb) was interpreted as being due to the presence of an extended repertoire of catabolic genes found on the *P. aeruginosa* PAO chromosome (Ratnaningshish et al., 1990). Although there is a large difference in genome sizes in different species, the variation among the different strains of species is usually within 20% (Drlica and Riley, 1990). Anagnostopoulous (1990) reported that *Bacillus subtilis* strains can have as much as 30% variation in genome size.

Similarly, the estimated genome size of *K. pneumoniae* M5a1 from this study is about 6% to 8% larger than the closely related enterics *E. coli* K12 and *S. typhimurium* LT2. An explanation for the large size of the *K. pneumoniae* genome is not readily available, but it is tempting to speculate that the pathogenic nature of *K. pneumoniae* coupled with its physiological ability are evidence for the presence of genetic information that is lacking in *E. coli* and *S. typhimurium*. Also, the presence of additional genes such as those which code for nitrogen fixation and capsular material may contribute to the increase in genome size. Further, the necessity for *K. pneumoniae* to adapt to varied environmental conditions, for

example, utilizing citrate as a sole source of carbon, may also result in the accumulation of extra genes. Characteristically *K. pneumoniae* is capable of uptake and catabolism of a wide range of nutrients not utilizable by *E. coli* and *S. typhimurium*. For example, it can produce acetoin from α-acetolactate and can decompose urea (Matsumoto, 1971). In addition, *K. pneumoniae* assimilates and respires nitrate (using it as an electron acceptor for anaerobic respiration). By contrast *E. coli* K12 and *S. typhimurium* LT2 do not assimilate nitrate during aerobic growth although both respire nitrate during anaerobic growth (Garzon et al, 1992). In this study, the calculated genome size difference between chromosomal fragments generated by a *Bln*I digest (5139 Kb $\pm$ 2.9%) and those generated from a *Xba*I digest (5103 Kb $\pm$ 2.7%) can be explained by the fact that differences in the observed genome size might merely reflect the differences in pulsed field gel electrophoresis conditions chosen in the study, as suggested by Krause and Mawn (1990). Further, I noticed that the lanes in each pulsed field gel electrophoresis are not always completely aligned. This may result in anomalous migration of the fragments (Maniloff, 1989), which would consequently lead to errors in fragment size estimation. In addition, it is noteworthy that a failure to identify doublets may lead to an underestimate of the genome size, or it is possible that smaller fragments may have run off the gel.

Most studies of prokaryotic genome topology show characteristically circular chromosomes. However, studies using pulsed field gel electrophoresis have revealed the existence of linear chromosomes in a few species (Davidson, 1992; Ferdows,

1989). When pulsed field gel electrophoresis was performed using M5a1 undigested chromosome, the DNA remained mainly within the gel plug at the wells and did not enter the gel. This observation is in agreement with the behavior of circular chromosomal DNA as reported by Krause and Mawn (1990) and Alleman et al, (1993), but does not match the expected behavior of linear DNA which should have migrated further into the gel.

The presence of DNA in bacterial cells is not necessarily limited to the chromosome. Plasmid DNA has been found in many strains of bacteria and sometimes the presence of particular plasmids are characteristic of particular bacterial species. The presence of even mega-sized plasmids (400-1500 Kb) has been reported for some species (Suwanto and Kaplan, 1989). I was able to find no evidence of plasmids in M5a1. However, when another strain of *K. pneumoniae* GM236 was analyzed, a megaplasmid was found (Lee Nansook, personal communication). The presence of plasmid DNA adds another dimension to the typing of these organisms. Plasmid typing may help to further categorize the different *K. pneumoniae* strains and it may be possible to relate the plasmid type to virulence in the case of *S. typhimurium*.

This genome map was established through reciprocal hybridization of *Xba*I fragments with *Bln*I fragments. When large fragments are isolated from the gels, they are generally mixed with small amounts of smaller fragments from other parts of the genome. The fragment clones used as probes often give rise to a major hybridization band due to the main fragment isolated and to many minor non-specific

bands due to the residual presence of the other fragments. This non specific hybridization has also been observed by Allardet-Servent et al, (1991) and by Lee et al, (1988). They explained it as being a consequence of random DNA degradation, causing purified genomic fragment probes to contain small amounts of fragments that are randomly broken from the rest of the chromosome.

There may be several ways to improve this condition:

(i) The same sample can be run more than once to check the position of the hybridized bands. The broken bands or any band that is not the original one will vary in migration but the real band will always be in the expected position.

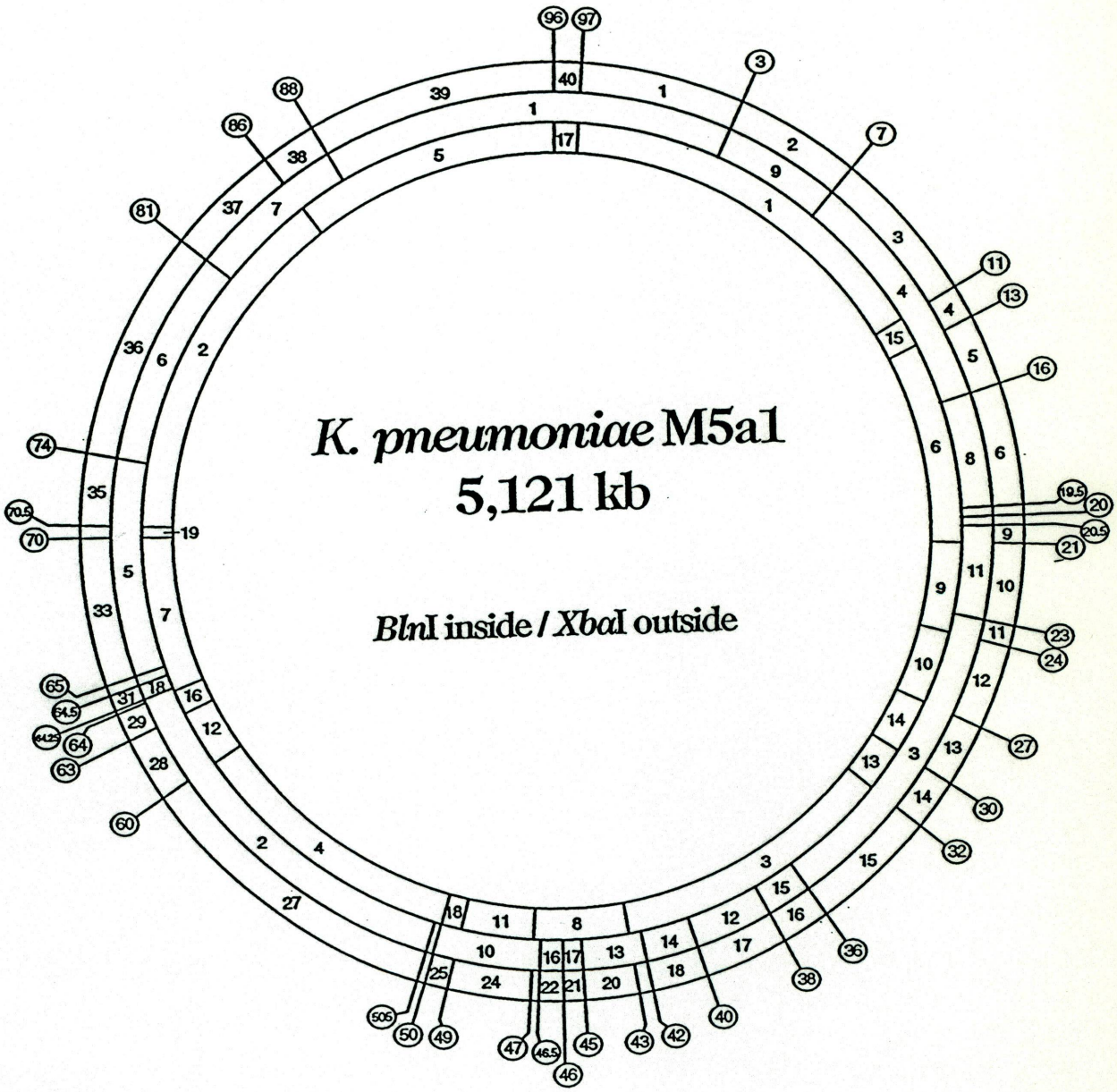
(ii) Back cross-hybridization with the detected band to the chromosome (cut with the restriction enzyme used to generate the probe), will define the primary hybridization fragments.

(iii) A third method to avoid background hybridization is to use small and specific probes to decide the linkage of a particular fragment. In this case a mini chromosomal library can be generated from either (a) a gel excised fragment or (b) by selecting the clones representative of the particular fragment from the entire chromosomal library (by using the fragment as a probe to the library). The fragment DNA from the mini-library can then be digested with rare cutting enzymes and linked to an antibiotic cassette (Michel and Cossart, 1992). This allows for the selection of the linking clones (those which carry rare cutting enzyme sites) on antibiotic supplemented media. These linking clones can then be used as small, specific probes against the digested genome.

An integral part of this project was the use of clones generated in this laboratory or acquired from other laboratories working with *K. pneumoniae* strains. The clones constructed in our laboratories are either random or nutritional. These known clones were used to approximate the location of specific genes. On the current map, I was able to localize these genes to the region of overlap between particular restriction fragments. However, this mapping does not allow the determination of the relative order of the genes mapped in each region or of the exact location of the gene within the region of overlap.

The approach I used to determine a range of minutes for the location of a gene is as follows: the *thr* gene was taken as representing the zero minute but the location of *thr* was assigned to the range 97-3 minutes (Fig 18). Therefore, I can say that the zero on this map can lie anywhere from 97 minutes to 3 minutes. This arrangement allows the rotation of the entire map in relation to the possible zero location. This map which shows a sectioned physical map, was used to define a range of locations for hybridized gene clones. Overlapping fragment regions formed by the *Xba*I and the *Bln*I restrictions were marked off and numbered as deduced new sections. The limits of each overlapping section were designated a map minute value. The entire chromosome was divided into 100 min as in the case of *E. coli* and *S. typhymurium*. The zero minute was taken as the center point of overlap section 1 (refer to Fig. 18) since this was the location of the *thr* gene. The hybridized gene clones were then assigned to the corresponding overlap section. The gene may fall anywhere within

Figure 18. An orientation map of M5a1. The outermost circle is this figure represents the overlap regions between the *Xba*I and *Bln*I generated fragments. The boundaries of each overlap fragment are marked by an approximate minute value (small circled numbers). The zero minute is taken as the center of the overlap fragment #1, which is the region where the *thr* clone hybridized.


K. pneumoniae M5a1
5,121 kb
BlnI inside / XbaI outside

the section and therefore the gene location is designated in a minute range which represents the limits of the overlap section.

Taking the gene location assigned into consideration, Table 4 was generated to compare the gene location on the *K. pneumoniae* chromosome to the already known *E.coli* map. Most gene locations correlate to the same location in *E.coli,* but some differences exist. The *rpoN* gene location for example is at 70 min in *E. coli* but was mapped at 97-3 min (Table 4) in *K. pneumoniae.* This 30 min difference would be very interesting if it is confirmed. However, it should be noted that the sigma 54 factor (product of *rpoN*) functions in trans and does not necessarily have to be linked to any genes it may affect. The *glnA*, *glnL* and *glnG* cluster codes for regulatory proteins which in *K. pneumoniae* affect the expression of the *nif* genes (MacFarlane and Merrick, 1985). Since *E. coli* does not fix nitrogen the location of these genes may differ between these two organisms in accordance to some beneficial organization for the cell.

The *narXL* genes in *K. pneumoniae* were estimated to be 74-81 min, but in *E.coli* the *narXL* genes are clustered at 27 min and are linked to *trp*. However, this linkage was not observed by others in *K. pneumoniae* (Stewart Valley, personal communication) which helped to confirm my results.

In the case of *phoE*, I detected three reasonably strong hybridization bands. These could be explained by the presence of 60% homology between *phoE*, *ompF* and *ompC* which code for other porins on the membrane (Van Der Ley et al., 1987). There can be several other reasons for the discrepancies observed between

TABLE 4. Correlation between gene locations on the *K. pneumoniae* and the *E.coli* chromosomal maps.

GENES	Number of overlapping fragments (outerlining in Fig. 18	Range of *K.pneumoniae* minute locations	*E.coli* minute location.
thr	1	97-3	1
rpoN	1	97-3	70
β-lactam	2	3-7	97
urease	3	7-11	NM
phoE	5,12,27	13,16 24-27 50-60	6,9,84,100
gdhA	6	16-19	27
KpnA	7	20	NM
nifJ,HDK	15	32-36	NM
type 3 *fimbrae*	15	32-36	NM
nac	15	32-36	NM
rcsA	27	50-60	NM
glnA, glnL, glnG	28	60-63	87
narXL	36	74-81	27
hut	37	81-86	NM
crp	39	88-96	74
β-gactosidase	1	97-3	8

NM = not mapped.

K. pneumoniae and *E. coli* as well. Genes having similar phenotypes can map to different locations in different genera. This could be due to either (i) homologousgenes that are actually at different location, or (ii)genes that may not share DNA homology although they have related or compensating functions.

When comparing this map to Matsumoto's genetic map (Matsumoto, 1971), I found that the *β gal* gene and the *nif* genes (location deduced by closeness to *his*) were at 93-3 and 26-32 min respectively. Similarly, *pur, pro* and *ural* were found to map at approximately 87-96, 3-7 and 7-11 min, respectively. These findings established that the map constructed here and Matsumoto's genetic map are in good agreement to a certain extent for the genes mapped on both. Correlation between the physical map and the genetic map is good in most cases. Some discrepancies may be due either to (i) polymorphism of restriction between strains used, or (ii) rearrangement of cloned DNA occurring during manipulation for gene cloning, especially when vector plasmid were used.

In this study, the *rcsA*, *nif* and *KpnA* genes were located on the map. These genetic markers are specific for *K. pneumoniae*. The *rcsA* gene codes for a polypeptide which is required for production of large amounts of capsular polysaccharide K-antigen (virulence factor) (McCallum and Whitfield, 1991). The *kpnA* gene was identified and cloned in our laboratory. This gene codes for a unique restriction enzyme which shows no homology to any of the *E. coli* or *S. typhimurium* genes (Valinluck, 1992).

Monnet et al (1990) reported on the difficulties of identifying *Klebsiella* strains

of clinical origin by the presently available system used in differentiation, such as DNA-DNA homology or plasmid profile analysis. To emphasize the importance of fingerprinting, Owen (1989) compared the sensitivity of DNA fingerprinting to other typing methods such as serotyping, bacteriophage typing, resistotyping, biotyping and ribotyping. They found that DNA fingerprinting was much more sensitive to subtle variations among strains than were the other methods.

I had predicted that strains obtained from the same clinical source (for example, urine) would exhibit similar fingerprinting patterns and differ from strains obtained from different locations (for example, lung). The rationale for this prediction is that the *K. pneumoniae* strains which would infect a particular organ must have the ability to recognize the tissue of that organ; for example, by having receptors which recognize markers on the cells of the organ. However, my results show variations within the *K. pneumoniae* cultures even when isolated from the same body cavity. One probable explanation for the diversity observed when the DNA fingerprint experiments were made using the *Xba*I enzyme is that the restriction activity of this enzyme is blocked by *dam* (DNA adenine methylation) and *dcm* (DNA cytosine methylation) methylation of the DNA (Allardet-Servent el al., 1988). Thus, similar strains which have different *dam* and *dcm* methylation patterns may in turn show different restriction pattern. Further studies to check this explanation would require the use of *dam*$^-$ and *dcm*$^-$ strains. Other explanations for the diverse of DNA fingerprints include chromosome arrangements such as translocation, inversion, insertion and deletion of chromosomal fragments.

Ankylosing spondylitis is an auto-immune disease that has similar diagnostic characteristics to rheumatism. The *K. pneumoniae* nitrogenase enzyme has a five amino acid identity overlap with the human HLA hyper-sensitivity factor. Due to this fact, nitrogen fixing *K. pneumoniae* has been implicated in the disease ankylosing spondylitis (Schwinimbeck and Oldstone, 1987). Although my project does not deal directly with this aspect of *K. pneumoniae*, it may be interesting to use the chromosomal fingerprint patterns as a means of tracking this pathogenic organism through a patient's infection history.

For future research in this *K. pneumoniae* genome project I would propose the identification of the true chromosome origin and terminus and track a replication forks moving along the chromosome in synchronously growing cells (Ohki and Smith, 1989), using simple pulsed field gel electrophoresis.

Since a general physical map has now been completed, further high resolution mapping can be achieved. Yoshida et al, (1993) reported a simple and efficient method for constructing high resolution physical maps. A specific gene can be targeted on the map and may be easily obtained from a pool of ordered clones. One particularly interesting study would involve molecular factors which contribute to pathogenicity. Similar to the approach used with *E.coli*, this physical map may be aligned with the genetic map. This would allow the quick and accurate mapping of new mutations after restriction analysis and fragment hybridization. Lastly, quoting Drlica and Riley (1990), "It is important to emphasize that there will not be a single

restriction map for a given strain of bacterium, much less for a given species, because differences occur within different isolates of a single strain".

APPENDIX

List of genome size analysis using PFGE.

Name	Size (Kb)	Physical map	# of genes mapped	References
Anabaena sp.strain PCC 7120	6,400	+	30	Bancroft et al., 1989
Anaplasma marginale	1,200	NA	NA	Alleman et al., 1993
Bacillus cereus	5,700	+	NA	Kolsto et al., 1990
Bacillus thuringiensis	5,400	+	NA	Carlso and Kolsto, 1993
Bacillus subtilus 168	4,165	+	NA	Itaya and Tanaka, 1991
Borrelia burgdorferi	946	+	3	Davidson et al., 1992
Borrelia burgdorferi B31	950	NA	NA	Ferdows and Barbour, 1989
Borrelia burdoferi, Sh-2-82	952	+	17	Casjens and Huang, 1993
Bradyrhizobium japonicum 110	8,700	+	63	Kunding et al., 1993
Brucella abortus 544^t	2,600	NA	NA	Allardet-Servent et al., 1988
Brucella melitensis 16 M Chromosome I ChromosomeII	2,100 1,150	+	8	Michaux et al., 1993
Campylobacter coli A417R	1,700	+	2	Yan and Taylor, 1991

Name	Size (Kb)	Physical map	# of genes mapped	References
Campylobacter coli	1,714	+	NA	Chang and Taylor, 1990
Campylobacter jejuni TGH9011	1,812	+	10	Kim et al., 1992
Campylobacter jejuni UA580	1,721	+	NA	Chang and Taylor, 1990
Campylobacter laridis UA487	1,451	+	NA	Chang and Taylor, 1990
Caulobacter crescentus	4,000	+	27	Ely et al., 1990
Chlamydia psittaci AB7, 1H, 1B	1,450	+	NA	Frutos et al., 1989
Chlamydia trachomatis L2	1,450	NA	NA	Frutos et al., 1989
Chlamydia trachomatis L2	1,045	+	13	Birkelund and Stephens, 1992
Clostridium perfrigenrs	3,600	+	24	Canard and Cole, 1989
Enterococcus faecalis	2,600	NA	NA	Bourgeois et al., 1989
Escherichia coli K-12	4,700	+	47	Smith et al., 1987
Haemophilus influenza type b	2,100	+	18	Butler and Moxon, 1990
Haemophilus influenza V23	1,980	+	NA	Kauc et al, 1989
Haemophilus influenza Rd	1,834	+	17	Lee et al, 1989

Name	Size (Kb)	Physical map	# of genes mapped	References
Haemophilus parainfluenza	2,340	+	NA	Kauc and Goodgal, 1989
Haloferax mediterranex	2,900	+	6	Lopez-Garcia et al., 1992
Haloferax volcanic DS2 1-chromosome 4 plasmids	2,920 690,442, 86,6.4	NA	NA	Charlebois et al., 1991
Lactococcus cremoris	2,600	NA	NA	Bourgeois et al., 1989
Lactococcus lactis	2,500	NA	NA	Bourgeois et al., 1989
Lactococcus lactis subsp. lactis DC11	2,580	+	6	Tulloch et al., 1991
Leptospira interoorgans 1-chromosome one plasmid	4,400 350	+	NA	Zuerner, 1991
Listeria monocyto gene	3,150	+	11	Michel and Cossart, 1992
Mycoplasma gallisepticum	1,050	NA	NA	Pyle et al., 1988
Mycoplasma genitalium	585	NA	NA	Su and Baseman, 1990
Mycoplasma hominis	704 to 825	NA	NA	Lagefoged and Christiansen, 1992
Mycoplasma hypopneumoniae	1,140	NA	NA	Pyle et al., 1988

Name	Size (Kb)	Physical map	# of genes mapped	References
Mycoplasma iowae	1,280	NA	NA	Pyle et al., 1988
Mycoplasma mobile	780	NA	NA	Bautsch, 1988
Mycoplasma pneumoniae	785	+	4	Krause and Mawn, 1990
Mycoplasma synoviae WVU 1853	900	NA	NA	Pyle et al., 1988
Mycoplasma mycoides *subsp. mycoides Y* *subsp. mycoides* GC 1176-2	1,240 1,330	NA	NA	Pyle et al., 1988
Myxococcus xanthus	9,454	+		Chen et al., 1991
Stigmatella aurantiaca	9,350	NA	NA	Neumam el al., 1991
Neisseria gonorrhoeae FA2090	2,219	+	11	Dempsey et al., 1991
Porochlamydia buthi	1,550	+	NA	Frutos et al., 1989
Porochlamydia chironomi	12,650	+	NA	Frutos et al., 1989
Pseudomonas aeruginosa	5,990	+	28	Romling et al., 1990
Rhodobacter capsulatus SB1003	3,600	+	22	Fonstein et al., 1992

Name	Size (Kb)	Physical map	# of genes mapped	References
Rhodobacter sphaeroides 2.4.1				
Chromosome I	3,046	+	20	Suwanto and Kaplan, 1989
Chromosome II	914	+	8	Suwanto and Kaplan, 1989
Rickettsiella grylli	2,100	+	NA	Frutos et al., 1989
Rickettsiella melolanthae	1,720	+	NA	Frutos et al., 1989
Salmonella typhimurium LT	4,807	+	102	Liu and Sanderson, 1992
Staphylococcus aureus	2,860	NA	NA	Weil and McLelland, 1989
Staphylococcus aureus 8325-4	2,748	+	1	Patel et al., 1989
Streptococcus mutans	6,715	+	NA	Okahashi et al., 1990
Streptococcus sanguis	2,300	NA	NA	Bourgeois et al., 1989
Streptococcus thermophilus	1,700	NA	NA	Bourgeois et al., 1989
Streptococcus coelicolor A3	8,000	+	37	Kieser et al., 1992
Streptomyces lividans 66	8,000	+	16	Leblond et al., 1993
Streptococcus pneumoniae	2,270	+	28	Gasc et al., 1991
Sulfolobus acidocaldarius	3,100	NA	NA	Yamagishi and Oshima., 1990
Sulfolobus acidocaldarius 7	2,760	NA	1	Kondo, 1992

Name	Size (Kb)	Physical map	# of genes mapped	References
Thermoautotrophicum Marburg	1,623	+	12	Stettler and Leisinger, 1992
Thermus thermophilus HB8	5,400	+	16	Borges and Bergquist, 1993
Thermus thermophilus HB 27	1,820	+	8	Tabata et al., 1993
Ureaplasma urealyticum 960^t	900	NA	NA	Cocks, et al., 1990

NA = not applicable
+ = physical map was constructed

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