PHYSICAL MAPPING AND PARTIAL CLONING OF *ACHLYA KLEBSIANA* MITOCHONDRIAL DNA

by

© David A. Boyd

a thesis presented to the

Faculty of Graduate Studies

University of Manitoba

In partial fulfillment of the

requirements for the degree

Master of Science

Department of Microbiology

October 1986

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read a Master's thesis entitled: Physical Mapping and Partial Cloning of Achlya klebsiana Mitochondrial DNA

submitted by David A. Boyd

in partial fulfillment of the requirements for the degree of Master of Science

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Date July 14, 1986

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

To my parents

To Brigitte

It's been a long time

for a little while

ACKNOWLEDGEMENTS

I would gratefully like to acknowledge friends, fellow students, and staff, whose assistance throughout the years was invaluable: ADAM, Gregor Awang, Oliver Bereznay, Sharon Berg, Dave Blicq, Thor Borgford, Sean Egan, Ron Eros, John Hickerson, Tom Hobman, Rob Hurta, Mike Logan, Sally McNabb, Mike Mulvey, Music, The Prep Ladies, Racquetball, Radio, Darren Raine, Danny Singh, Satnam Singh, Six Months Off, Mark Smolenski, Jack Switala, Debby Teich, Jaunita Thiessen, Barb Triggs-Raine, Canoe Trips, UMZOO, and Ingemar Von Ossowski. Special thanks to Sally for the A^+ help in restriction mapping pMAK5 and pDAB6.5.

Special thanks to Dr. H. Duckworth, Dr. H.B. LeJohn, and Dr. P. Loewen, for helpful discussions and use of equipment and materials.

Last but not least, heartfelt thanks to Dr. Glen Klassen for his friendship, wisdom, patience, and money.

ABSTRACT

A physical map of the mtDNA of *Achlya klebsiana* has been constructed. The mt genome is a circular molecule of 50.4 kb. A total of 71 restriction sites for 15 enzymes have been mapped. A large portion of the mt genome exists in the form of an inverted repeat, each repeat region being from 9.68 to 12.45 kb in length. The repeat regions are separated by single copy sequences which are present in head-to-head or head-to-tail orientations. These two flip-flop isomers are present in approximately equal abundance in the population of DNA molecules. Two restriction fragments of the mtDNA have been cloned into plasmid vectors. One cloned fragment originates wholly from the repeat regions while the other contains DNA from both repeated and unique sequences. The two cloned fragments share about 3.7 kb of common sequence. The physical maps of *A. klebsiana* and *A. ambisexualis* were compared and although they are essentially colinear, *A. klebsiana* appears to contain an insertion of 0.4-0.7 kb in the repeat region relative to the analogous region of *A. ambisexualis*. Analysis of sequence divergence between the two species indicates that base substitutions in *Achlya* are non-random, the inverted repeat evolving more slowly than the single copy DNA.

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LIST OF ABBREVIATIONS

A	deoxyadenosine
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
C	deoxycytidine
CIAP	calf intestinal alkaline phosphatase
cm	centimeters
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol (Cleland's reagent)
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine-tetra-acetic acid
fig(s)	figure(s)
G	deoxyguanosine
g	gram(s)
h	hour(s)
IPTG	isopropyl-beta-D-thio-galactopyranoside
kb	kilobase pairs
kDa	kilodalton(s)
L	liter(s)

M	molar
MOPS	morpholinopropanesulfonic acid
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar
mt	mitochondrial
mtDNA	mitochondrial DNA
m.u.	map units
uCi	microcuries
ug	microgram(s)
uL	microliter(s)
um	micrometer(s)
ng	nanogram(s)
nm	nanometer(s)
O.D.	optical density
pmoles	picomole(s)
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate

T	deoxythymidine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
V	volts
vol	volume(s)
X-gal	5-bromo-4-chloro-indolyl-beta-galactoside

INTRODUCTION

INTRODUCTION

Mitochondrial genomes are functionally conservative, encoding basically the same genes in all eukaryotes. These include the components of a distinctive mt protein-synthesizing system whose purpose is to translate a limited number of mtDNA-encoded messenger RNAs (Wallace, 1982; Gray, 1982; Dujon, 1983). In contrast to this functional conservatism, mt genome structure and organization are highly diverse. Except for the metazoa, mtDNAs are highly variable in size and hence in potential genetic information content. Mitochondrial gene order is the same in closely related organisms but differs in more distantly related ones.

Among fungi, information concerning mt genome organization is primarily derived from studies of the Ascomycotina. There is a six to seven-fold variation in mt genome size (17-115 kb) and gene order is highly variable. Among zoosporic fungi (Mastigomycotina), information is limited to physical maps for the chytrid Allomyces (Borkhardt and Delius, 1983) and the oomycetes Phytophthora (Klimczak and Prell, 1984) and Achlya (Hudspeth et al, 1983; Boyd et al, 1984; Shumard et al, 1986). Restriction mapping of the heterothallic A. ambisexualis E-87 revealed that the mtDNA contains a large inverted repeat (Hudspeth et al, 1983).

Large inverted repeats are typically found in chloroplast DNAs but are relatively rare in mtDNAs (Palmer, 1985). Preliminary restriction mapping of the homothallic A. klebsiana mtDNA confirmed the findings for E-87 and thus suggest that the chloroplast-like organization is general for Achlya species (Boyd et al, 1984). Indeed, mapping of mtDNA from three other heterothallic Achlya strains revealed similar organizations (Shumard et al, 1986). Comparison between the maps of the four heterothallic strains showed that the Achlya mt genome is subject to insertion/deletion events, and in addition, analysis of sequence divergence between strains showed that the repeat DNA sequences are evolving more slowly than are the single copy sequences (Shumard et al, 1986).

The availability of a detailed physical map of A. klebsiana mtDNA not only will add to the limited information concerning mt genome organization among zoosporic fungi, but will allow comparisons with the more distantly related heterothallic Achlya strains. Comparisons between restriction maps will reveal whether the homothallic A. klebsiana mtDNA contains any of the insertion/deletions of the heterothallic Achlya mt genomes, and if the relative conservation of repeat sequences is still retained in a pairwise comparison between A. klebsiana and a heterothallic Achlya strain.

HISTORICAL

HISTORICAL

1. The petite mutation of yeast and the discovery of mtDNA

The discovery of the cytoplasmically inherited petite mutation of yeast (Ephrussi et al, 1949a, 1949b) set the stage for the study of the mt genome, although at the time mtDNA had yet to be discovered.

Ephrussi and his collaborators were interested in understanding the petite mutation, so called because although the mutation abolished the capacity of yeast (Saccharomyces cerevisiae) to grow on non-fermentable substrates such as ethanol or glycerol, petite mutants could still grow on glucose and other fermentable sugars, forming small colonies.

The first petite mutation (later to be called a neutral petite) was inherited in a non-Mendelian fashion and so was considered to be cytoplasmic in origin (Ephrussi et al, 1949b). More evidence that the neutral petite mutation was cytoplasmic came with the discovery of a spontaneously occurring petite that was the result of a nuclear gene mutation with normal Mendelian inheritance characteristics (Chen et al, 1950). When the nuclear petite was mated with the neutral petite the result was a diploid yeast with a normal phenotype (large colonies), which upon sporulation showed normal Mendelian segregation in the

four spores of the asci (2:2 ratio). The interpretation was that the neutral petite had a normal wild type nuclear gene but exhibited the petite phenotype due to a cytoplasmic mutation, whereas the nuclear petite exhibited the petite phenotype due to a mutation in a nuclear gene. The diploid yeast was heterozygous for the mutant nuclear gene and had a normal cytoplasm, and so showed a normal phenotype. A third type of petite found in Ephrussi's laboratory was called the suppressive petite (Ephrussi et al, 1955). Matings between normal yeasts and suppressive petites gave rise to cultures in which the normal phenotype was suppressed, hence the name. Sub-cloning of a suppressive petite gave an array of subclones with different degrees of suppressiveness (Ephrussi and Grandchamp, 1965; Ephrussi et al, 1966).

By this time mtDNA had been discovered in chick embryos (Nass and Nass, 1963a, 1963b) and evidence for DNA in yeast mitochondria was being reported (Schatz et al, 1964; Yotsuyangi and Guerrier, 1965; Yotsuyangi, 1966). A relationship between mtDNA and the petite phenotype of yeast was established when it was shown that mtDNA from petite mutants and from normal yeasts differed greatly in buoyant densities (Mounolou et al, 1966). Although Ephrussi himself never mentioned mtDNA as a possible site for the neutral petite mutation, his investigations of cytoplasmic inheritance set the

stage for further research into the structure and function of yeast mtDNA (Roman, 1980, 1982).

The studies of Ephrussi and the discovery of mtDNA marked the beginning of a growing interest in the function of the mt genome and the organization and expression of its genes. It was found that many petites are deletion mutants in which more than 50% of the wild type mtDNA has been removed (Borst and Grivell, 1978). Some mutants retain only 0.1% of the complexity of the wild type mt genome. The size of the mtDNA in petites is equal to that in wild type due to amplification of the remaining segment. This results in molecules with tandem duplications. The repetitive mtDNA molecules of petites are replicated faithfully without major errors and thus provide a natural method for cloning DNA segments. Petite strains of yeast can still mate with wild type cells and transfer the genetic markers in their mtDNA to wild type mtDNA, thus allowing genetic characterization of the mtDNA segments retained in the petite mutant. A genetically characterized petite mtDNA molecule can be used as a probe to find homologies in other organisms (Agsteribbe et al, 1980). Thus, the petite yeast has had and continues to have an important role in the elucidation of mt genome organization.

2. Physical diversity of mtDNAs

Mitochondrial DNAs are diverse in both size and shape (Wallace, 1982; Gray, 1982; Dujon, 1983). All metazoans, from flatworms to man, possess a mt genome that consists of a circular molecule ranging in size from 15 to 19 kb (Altman and Katz, 1976). Among fungi, mt genome size variation is extensive, ranging from about 17 kb for a strain of Schizosaccharomyces pombe, (Zimmer et al, 1984) to 115 kb for Cochliobolus heterostrophus (Garber and Yoder, 1984). The mt genome of higher plants is much larger and even more variable than that of fungi (Leaver and Gray, 1982; Leaver et al, 1983). Estimates of the size of plant mtDNAs range from 200 kb in Oenothera (Brennicke, 1980) and Brassica (Palmer and Shields, 1984; Chetrit et al, 1984) to 2500 kb in muskmelon (Ward et al, 1981). Much of the size variation in mtDNA is due to the presence of introns and non-coding intergenic regions. In fungal mtDNAs, genes may be separated by non-genetic DNA and many of them contain introns (see Historical section 3.5). The introns may be facultative such that there can be considerable size variation even among closely related species. In higher plants, larger mtDNA size may be due to additional coding functions (see Historical section 3.4) as well as the presence of 'promiscuous' chloroplast DNA (Stern and Lonsdale, 1982; Stern and Palmer, 1984a). Metazoan mtDNAs contain no

introns, have little or no intergenic spaces, and even exhibit gene overlap (see Historical section 3.2).

Circular mtDNAs predominate and are found in all metazoa and in the vast majority of fungi, algae, and protozoa. Linear mtDNAs have been found in the ciliated protozoans Tetrahymena (Goldbach et al, 1979) and Paramecium (Goddard and Cummings, 1977), the yeasts Hansenula mrakii (Wesolowski and Fukuhara, 1981) and Candida rhagii (Kovac et al, 1984), the slime mold Physarum polycephalum (Kwano et al, 1982), and the green alga Chlamydomonas reinhardtii (Ryan et al, 1978). Trypanosomes contain an unusual mtDNA in their single mitochondrion (kinetoplast) consisting of a network of catenated minicircles and maxicircles (Englund et al, 1982). The minicircles (1-3 kb) have no known function, whereas the maxicircles (20-40 kb) are the informational mtDNA (see Historical section 3.3). In higher plants it has been diffuclut to determine genome organization because restriction enzyme patterns are complex and electron microscope analysis reveals linear and circular molecules of various sizes (Leaver and Gray, 1982). Recently however, the plant mt genome has been viewed as a set of circular molecules of different sizes, each member of the set being a subset of a master circular sequence (Lonsdale, 1984). Circular maps have been published for Brassica and Zea mays (see Historical section 3.4).

3. Mitochondrial genes and their organization

3.1 Introduction

In contrast to the vast structural variation of mt genomes, all encode a small number of ubiquitous polypeptides that are involved in respiration. These include subunits 1, 2, and 3, of cytochrome c oxidase (COI, COII, COIII), apocytochrome b (cyt b), subunits 6 and 8 of the Fo-ATPase (ATPase 6 and ATPase 8) and, depending on the organism, subunit 9 of the Fo-ATPase (ATPase 9). Various mt genomes also encode other polypeptides as discussed below. All mt genomes also encode the RNA molecules involved in mt protein synthesis, namely the small and large rRNAs and 22-28 tRNAs. Plant mtDNA also encodes a 5S rRNA (see Historical section 3.4) and protozoa may encode fewer tRNAs (see Historical section 3.3). Mitochondrial translation uses a simplified mechanism for reading the genetic code, thus minimizing the number of tRNA species required (reviewed in Breitenberger and RajBhandary, 1985). Organisms in every major mt system studied - mammal, insect, amphibian, fungal, protozoan, and plant - deviate from the nuclear genetic code in one way or another such that there is no non-standard codon dictionary common to all mitochondria (Breitenberger and RajBhandary, 1985).

3.2 Metazoan mtDNA

The complete nucleotide sequences of the human (16,559 bp; Anderson et al, 1981), bovine (16,338 bp; Anderson et al, 1982), mouse (16,295 bp; Bibb et al, 1981), Drosophila yakuba (16,019 bp; Clary and Wolstenholme, 1985), and Xenopus laevis (17,553 bp; Roe et al, 1985) mt genomes have been reported. All have the same gene complement (COI, COII, COIII, cyt b, ATPase 6,ATPase 8, two rRNAs, and 22 tRNAs). The open reading frame originally designated URFA6L in metazoan mtDNAs has been shown to have amino acid sequence homology to the aap1 gene (ATPase 8) of S. cerevisiae and hence has been renamed ATPase 8 (Macreadie et al, 1983). In addition, the metazoan mtDNAs contain seven unidentified reading frames (URFs) designated URF1, URF2, URF3, URF4, URF4L, URF5, and URF6. The products of six of these URFs (URF1, URF2, URF3, URF4, URF4L, and URF5) have recently been identified as components of the respiratory chain NADH dehydrogenase (Chomyn et al, 1985). The identity of the URF6 product is unknown. The overall gene organization is identical in all vertebrates, with nearly all of the size difference between the mammalian and amphibian mt genomes due to a significantly larger (approximately 1 kb) displacement loop (D-loop) region in amphibians. Invertebrates, represented here by Drosophila yakuba, although they have a mt gene complement that is identical to that of

the vertebrate mt genomes, have a different overall gene order (Clary and Wolstenholme, 1985). The D-loop region of all the metazoan mtDNAs contains the origin of replication of the heavy (H) strand (major coding strand), and, in the case of the mammalian mtDNAs, transcription of both heavy and light (L) strands is thought to be initiated in this region (Clayton, 1984). In vertebrates the origin of replication of the light strand is in a hairpin structure located in a cluster of five tRNA genes between COI and URF2. No analogous structure is found in the corresponding region or in any other intergenic region of Drosophila yakuba.

The most striking feature of the metazoan mtDNAs is their highly compact and efficient organization. Genes are tightly packed with few or no non-coding nucleotides between them, and do not contain a single intron. In fact, all the sequenced metazoan mtDNAs contain examples of gene overlap. For example, the ATPase 8 and ATPase 6 reading frames overlap by 7 bp in Drosophila, 10 bp in Xenopus, 40 bp in bovine, 43 bp in mouse, and 46 bp in human. The tRNA genes are distributed throughout the metazoan mt genome and flank other genes at one and usually both ends. The tRNA genes are thus thought to act as 'punctuation marks' in processing of the major polycistronic RNA transcripts (Ojala et al, 1981). Following cleavage from the primary transcript, pre-mRNAs without a complete

translation stop codon (those ending in a U or UA) are polyadenylated to generate a UAA stop codon (Anderson et al, 1981). In addition, the pre-tRNAs undergo post-transcriptional addition of CCA to their 3' terminus to form mature, functional species.

The metazoan mt genomes are so compact that, with the exception of the D-loop region, the mtDNA consists almost totally of coding regions. The total number of intergenic nucleotides is only 183 in Drosophila yakuba, 87 in human, 64 in mouse and Xenopus laevis, and 57 in bovine mtDNA.

3.3 Protozoan mtDNA

Among the few linear mtDNAs found so far, two are in ciliated protozoans. Tetrahymena pyriformis contains a 43 kb linear mtDNA (Goldbach et al, 1979), and Paramecium aurelia contains a 42 kb linear mtDNA (Goddard and Cummings, 1977). In Tetrahymena mtDNA there are two copies of the large rRNA gene, one near each terminus of the molecule, in opposite orientations. The small rRNA gene is located approximately 12 kb from one end of the molecule. Interestingly, 36 tRNA species have been isolated from the mitochondria of Tetrahymena, but only 10 tRNAs appear to be coded for by the mtDNA (Suyama, 1986). In contrast, metazoan and fungal mtDNAs encode 22-28 tRNAs (Historical sections 3.2 and 3.5).

Seven tRNA gene loci have been mapped on the Tetrahymena mt genome (Suyama et al, 1985) and six of the tRNA genes have been sequenced (Suyama, 1985; Suyama et al, 1985).

In Paramecium mtDNA there is a single large rRNA gene located near one end of the molecule. The small rRNA gene is located approximately 10 kb from the large rRNA gene. Both rRNA genes have been sequenced, as has a $tRNA^{tyr}$ gene which follows the 3' end of the large rRNA gene (Seilhamert et al, 1984a, 1984b).

The minicircle DNA of the kinetoplast DNA of trypanosomes is heterogeneous in sequence, evolves rapidly, is not transcribed, and has no known function. The maxicircle DNA of the kinetoplast DNA is homogeneous in sequence, conserved in evolution, is transcribed, and is the informational mtDNA. Maxicircle DNA ranges in size from 20 to 40 kb depending on the organism, with most of the size variation due to different species-specific lengths of the non-transcribed region. The transcribed region of maxicircle DNA is constant in size between species (16-17 kb), and DNA sequencing in two organisms has confirmed that it is the informational mtDNA. Approximately 70% of the 22.5 kb Trypanosoma brucei maxicircle DNA has been sequenced, and this represents about 90% of the transcribed region (Eperon et al, 1983; Benne et al, 1983a, 1983b; Hensgens et al, 1984).

The genes for the small and large rRNAs, COI, COII, and cyt b, have been identified. In addition, ten URFs have been identified with three of them having distinct homology to mammaliam URF1, URF4, and URF5. Approximately 45% of the 30 kb Leishmania tarentolae maxicircle DNA has been sequenced, and this represents about 80% of the transcribed region (de la Cruz et al, 1984, 1985a, 1985b). The genes for the small and large rRNAs, COI, COII, COIII, and cyt b, have been identified. In addition, twelve URFs have been identified with two of them having distinct homology to mammalian URF4 and URF5. The transcribed regions of both T. brucei and L. tarentolae appear to be essentially colinear. However, no COIII gene has been identified in T. brucei. The conclusion is that the COIII gene is absent from the T. brucei maxicircle DNA or is extensively diverged in sequence. Surprisingly, no tRNA genes have been identified so far in either T. brucei or L. tarentolae maxicircle DNAs, and the genes for ATPase 6, ATPase 8, and ATPase 9 have not been located. Although the gene order of the trypanosome maxicircle DNA is different from that of the metazoans, the transcribed region is of a similar size (16-17 kb) and shows the same genetic compactness. No introns are present and several of the reading frames overlap.

3.4 Plant and algal mtDNA

As mentioned above (section 2), the physical diversity of plant mtDNA has led to difficulties in constructing physical maps of plant mt genomes. Recently however, physical maps of the 218 kb Brassica campestris (Chinese cabbage, turnip) mtDNA (Palmer and Shields, 1984), of the 217 kb Brassica oleracea (cauliflower) mtDNA (Chetrit et al, 1984), and of the 570 kb Zea mays (maize; N fertile cytoplasm) mtDNA (Lonsdale et al, 1984) have been published. Based on the structures of the Brassica and maize mt genomes, a general structure for higher plant mtDNA has been proposed (Lonsdale, 1984). The plant mt genome can be arranged into a single circular master chromosome which bears the entire sequence complexity of the genome. The master chromosome will contain inverted and/or directly repeated sequences between which homologous intragenomic recombination occurs. Recombination between direct repeats on the master molecule gives rise to sub-genomic molecules by a reversible 'loop-out' mechanism. The number and location of direct repeats will determine how the mt genome can be subdivided. The relative molarities of the various circles will depend on the rates of both intra- and intermolecular recombination between homologous repeats. In Brassica campestris, recombination between the major 2 kb direct repeats on the 218 kb master molecule

produces two smaller circles - 135 kb and 83 kb sub-genomes (Palmer and Shields, 1984). The data from Brassica oleracea are consistent with the Brassica campestris model, although in cauliflower the major repeat elements are located on the master molecule such that recombination between them would result in 172 kb and 45 kb sub-genomes (Chetrit et al, 1984). In both Brassica species there appear to be other minor repeat elements present and homologous recombination between them leads to other distinct minor sub-genomic circles, thus accounting for the heterogeneity found in the Brassica mt genome. In maize, the mt genome exists primarily as 67 kb and 503 kb circles which can integrate with one another by intermolecular homologous recombination between 12 kb direct repeats, to form the 570 kb master molecule (Lonsdale et al, 1984). Further, the 503 kb sub-genome can be subdivided into two circles of 250 kb and 253 kb by recombination between a pair of 3 kb direct repeats. In addition, the master molecule contains 1, 2, and 10 kb direct repeats, and a 14 kb inverted repeat. As in Brassica, secondary recombination between minor repeat elements occurs, leading to other minor sub-genomic circles. Repeat elements that seem to be involved in recombination have also been found in the mt genomes of wheat (Falconet et al, 1984, 1985) and in spinach and two species of pokeweed (Stern and Palmer, 1984b).

Mitochondrial protein analysis has indicated that COI, COII, ATPase 9, and cyt b, are mt gene products in maize (Leaver et al, 1983; Hack and Leaver, 1983), and that the F1-ATPase subunit alpha of maize (Hack and Leaver, 1983) and of Vicia faba (Boutry et al, 1983) are mt gene products. The encoding of the F1-ATPase subunit alpha is the first example of an additional coding function for plant mitochondria as compared to other mt systems. In addition, a 60-62 kDa heat shock protein is encoded within the mitochondria of maize and Brassica (Sinibaldi and Turpen, 1985). DNA sequence analysis has led to the identification of the genes for some of these products in maize and in other plants. In maize, the genes for COI (Isaac et al, 1985a), COII (Fox and Leaver, 1981), ATPase 9 (Dewey et al, 1985), cyt b (Dawson et al, 1984), and the F1-ATPase subunit alpha (Isaac et al, 1985b), have been sequenced. All of the sequenced genes are continuous except for COII, which contains a single 794 bp intron. There are two copies of the gene for F1-ATPase subunit alpha in the male fertile maize mt genome (Zea mays L.), whereas male sterile mt genomes contain only one copy (Isaac et al, 1985a). The COII gene has also been sequenced in Oenothera (Hiesel and Brennicke, 1983), wheat (Bonen et al, 1984), rice (Kao et al, 1984), and pea (Moon et al, 1985). The COII genes of the monocotyledons rice and wheat contain an intron at exactly the same

position as that in the COII gene of maize, whereas the COII genes of the dicotyledons pea and Oenothera are continuous. The COII gene in Oenothera overlaps by four nucleotides an open reading frame of 177 bp that codes for a protein of 58 amino acids with structural homology to the ATPase 8 of S. cerevisiae (Hiesel and Brennicke, 1985). The cyt b and $tRNA^{fmet}$ genes have been sequenced in wheat (Boer et al, 1985b; Gray and Spencer, 1983) and Oenothera (Schuster and Brennicke, 1985; Gottschalk and Brennicke, 1985).

The first genes to be directly identified on plant mtDNA were those for the rRNAs of wheat (Bonen and Gray, 1980). It was found that 26S, 18S, and 5S mt rRNAs were encoded by wheat mtDNA, and that the 18S and 5S rRNA genes were closely linked but physically distant from the 26S rRNA gene. Subsequently, the rRNA genes in maize mitochondria were mapped and a similar organization was found (Stern et al, 1982). This arrangement of mt rRNA genes (closely linked 18S and 5S rRNA genes physically distant from the 26S rRNA gene) seems to be a common feature of higher plants (Huh and Gray, 1982). Of all mt systems studied, only in plant mitochondria has a 5S rRNA species been found (Leaver and Harmey, 1976). In maize the 26S rRNA gene (Dale et al, 1984) and the 5S and 18S rRNA genes (Chao et al, 1983, 1984) have been sequenced. The 26S rRNA gene (Manna and Brennicke, 1985) and 5S and 18S rRNA genes

(Brennicke et al, 1985) have also been sequenced in Oenothera. The 18S and 5S rRNA genes have been sequenced in wheat (Spencer et al, 1981, 1984), and soybean (Morgens et al, 1984; Grabau, 1985). In wheat mtDNA the 18S and 5S rRNA genes are located on one repeated DNA segment, while the 26S rRNA gene is located on another, and thus all are present in multiple copies (Falconet et al, 1984, 1985). In maize the genes for COI, COII, cyt b, F1-ATPase subunit alpha, and the rRNAs, have been located on the physical map of the mtDNA (Dawson et al, 1986).

In contrast to the large and complex mt genomes of higher plants, the mt genome of Chlamydomonas, a green alga, is relatively simple. C. reinhardtii contains a 16 kb linear mtDNA (Ryan et al, 1976). Hybridization with heterologous gene probes has allowed identification of regions on the C. reinhardtii mtDNA encoding cyt b, COI, and the small and large rRNA genes (Boer et al, 1985a). Sequence analysis has identified the genes for COI and genes homologous to mammalian URF2 and URF5 (Pratje et al, 1984; Boer et al, 1985a; Vahrenholz et al, 1985; Boer and Gray, 1986). Little is known concerning the mt genomes of other algae. However, it is interesting to note that in the unicellular, heterotrophic, achloric alga Prototheca zopfii, the F1-ATPase subunit alpha is encoded within the mitochondria (Deters and Ewing, 1985).

3.5 Fungal mtDNA

Early studies on mt genome organization naturally focused on that in S. cerevisiae. Both biochemical and genetic approaches were used to construct genetic and physical maps of several yeast strains (Borst and Grivell, 1978). The maps constructed showed that S. cerevisiae contains a circular mtDNA of about 70 to 80 kb depending on the strain. The gene order among different strains was found to be conserved, although restriction enzyme analysis revealed many minor size variations (point mutations and insertions/deletions of ± 25-50 bp) and some major size variations (insertions/deletions of up to 3 kb) in the mtDNAs of different strains. Since the publication of the first genetic and physical maps, primary structural data on the S. cerevisiae mt genome have been accumulating such that the sequence of 87% of the mt genome is available (deZamaroczy and Bernardi, 1985). Compilation of all data has led to a new estimate of mt genome sizes for a number of S. cerevisiae strains. The new sizes are about 9% to 12% higher than previous estimates and range from 74 to 85 kb. About one-third of the mt genome consists of coding sequences while the remaining two-thirds consists of non-coding A+T rich regions. DNA sequence data exist for COI, COII, COIII, cyt b, ATPase 6, ATPase 8, ATPase 9, the large (21S) and small (15S) rRNAs, and 24 tRNAs. Also sequenced is

the gene for the mito-ribosomal protein var1 and a locus whose RNA product is necessary for synthesis of the mt tRNAs. Sequence data from intergenic regions includes that for seven replication origins (ori/rep) and five open reading frames whose products are unknown. None of the open reading frames show homology to the mammalian mt URFs. Three genes, COI, cyt b, and 21S rRNA, are interrupted by introns in most strains. The presence or absence of some of these introns accounts for many of the interstrain differences in mt genome size. In the longest mt genomes (85 kb), the COI gene contains nine introns, the cyt b gene five introns, and the 21S rRNA gene one intron. Short mt genomes (78.5 kb) do not contain introns aI5-alpha and aI5-beta in the COI gene, and introns bI1, bI2, and bI3 in the cyt b gene. The super-short mt genome (74 kb) of S. carlbergensis, does not contain introns aI1, aI4, aI5-alpha, and aI5-beta in the COI gene, introns bI1, bI2, and bI3 in the cyt b gene, and the 21S rRNA gene intron. The introns in yeast mtDNA can be assigned to two families (Group I and Group II) based on similarities in nucleotide sequence such that members of the same family share short but distinctive sequence stretches between which interactions occur causing introns to fold into a secondary structure necessary for splicing out of the intron (Michel and Dujon, 1983; Waring and Davies, 1984). Members of both

families may contain open reading frames (ORFs). In some cases, genetic analysis has shown that the intronic ORFs encode proteins (maturases) that are necessary for correct processing of the precursor mRNA for the gene containing the intron (Grivell, 1983). Some introns are capable of self-splicing in the absence of protein. These include intron aI5-gamma (Group II, no ORF) of the COI gene (Peebles et al, 1986; Van der Veen et al, 1986), and the 21S rRNA intron (Group I, contains an ORF) (Van der Horst and Tabak, 1985). The 21S rRNA intronic ORF encodes a protein that is required for transposition of the intron itself into intronless 21S rRNA genes that occur in crosses between intron-plus and intron-minus yeast strains (Jacquier and Dujon, 1985; Macreadie et al, 1985).

As more information became available concerning mt genes in *S. cerevisiae*, interest in the mtDNA of other fungi increased. Identification of mt genes in other fungi was done using genetically defined petite mtDNAs as gene probes to find homologies in other organisms. Most of these studies centered on the ascomycetes. Among ascomycetous yeasts, mt gene maps of varying complexity have been constructed for *Hansenula mrakii* (Wesolowski and Fukuhara, 1981), *Kluyveromyces lactis* and *Saccharomycopsis lipolytica* (Wesolowski et al, 1981), *Saccharomyces exiguss* (Clark-Walker et al, 1983), *Hansenula petersonii* (Falcone, 1984),

Schizosaccharomyces pombe (Lang and Wolf, 1984), and Eeniela nana and six species of Dekkera/Brettanomyces (Hoeben and Clark-Walker, 1986). Among the imperfect fungi (Deuteromycotina), mt gene maps have been constructed for the yeasts Kloeckera africana and Torulopsis glabrata (Clark-Walker and Sriprakash, 1981, 1983). Among filamentous ascomycetes, mt gene maps have been constructed for Neurospora crassa (Macino, 1980; Agsteribbe et al, 1980), Aspergillus nidulans (Macino et al, 1980), and Podospora anserina (Kück and Esser, 1982; Wright et al, 1982; Jamet-Vierny et al, 1984).

Partial DNA sequence data from the 18.9 kb mtDNA of T. glabrata have led to identification of all its tRNA genes and the ATPase 8 gene (Clark-Walker et al, 1985), and a gene homologous to the S. cerevisiae var1 gene (Ainley et al, 1985).

Partial DNA sequence data are available for the 94 kb mtDNA of P. anserina (Jamet-Vierny et al, 1984; Osiewacz and Esser, 1984; Cummings et al, 1985). Completely sequenced genes include those for ATPase 8, five tRNAs, and three URFs which are not homologous to any of the mammalian mt URFs. Partial sequence data are available for the 5' and 3' ends of the COI gene, including the first intron. The DNA sequence data show that the COI gene is highly mosaic, the 5' and 3' termini being about 20 kb apart.

Partial sequence data for a gene homologous to the mammalian mt URF1, indicate that the gene is mosaic, containing at least three introns.

Considerable sequence data have accumulated for the mt genomes of *N. crassa*, *A. nidulans*, and *S. pombe*.

For *N. crassa*, approximately 80% of the mt genome has been sequenced (Breitenberger and RajBhandary, 1985). Among the ubiquitous mt polypeptides, complete sequences are available for COI, COII, COIII, ATPase 6, ATPase 8, ATPase 9, and cyt b. In addition, URFs homologous to mammalian mt URF1, URF2, URF5, and URF6, have been identified. Several other URFs not homologous to any of the mammalian mt URFs have also been identified. The small (17S) rRNA gene and more than 90% of the large (24S) rRNA gene have been sequenced. A total of 27 tRNA genes have been sequenced, two of which are duplicated. Thus, there is a total of 25 different tRNA species in the mtDNA. The COI gene in the common laboratory *N. crassa* strain from which the sequence was obtained, is not interrupted by introns. However, several wild-type strains of *N. crassa* have been found to contain up to four introns in the COI gene (Collins and Lambowitz, 1983). The cyt b gene contains two introns (both Group I), which are found at sites different from any of those of introns in the *S. cerevisiae* cyt b gene. The first intron has no ORF and has been shown to be self-splicing *in vitro*

(Garriga and Lambowitz, 1984). The second intron contains an ORF that could code for a maturase. The ATPase 6 gene contains two introns, the first being short (93 bp), containing no ORF, and the second being a Group I intron that could code for a maturase. The URF1 gene contains one intron (Group I) that has an ORF, but the intron appears not to be related to those encoding maturases. The presence of an ATPase 9 gene in the mtDNA was unexpected since it had been shown that the N. crassa ATPase 9 subunit is encoded in the nucleus (Sebald et al, 1979). It is not known whether the mt ATPase 9 gene is completely silent or not. The large (24S) rRNA gene contains one intron (Group I) which is located at exactly the same site as the intron in the corresponding gene of S. cerevisiae. The intron contains an ORF which is not homologous to the S. cerevisiae intronic ORF, and that appears to be the gene for the mito-ribosomal protein S5, known to be encoded in the mitochondria of N. crassa. Virtually every gene in N. crassa mtDNA is flanked by highly conserved GC rich palindromic sequences, most of which contain two closely spaced PstI sites. These PstI palindromes may represent 5-10% of the mtDNA. Despite punctuation of the mt genes in N. crassa by PstI palindromes, the palindromes do not appear to function in processing of polycistronic mRNAs.

For A. nidulans, more than 97% of the mt genome has been sequenced (Brown et al, 1985). The ubiquitous mt polypeptides, COI, COII, COIII, ATPase 6, ATPase 8, ATPase 9, and cyt b, have been completely sequenced. Reading frames homologous to mammalian mt URF1, URF2, URF3, URF4, and URF5 have been identified, as have several other URFs whose products are unknown. Both small (16S) and large (23S) rRNA genes have been sequenced, as has a total of 27 tRNA genes. Two of the tRNA genes are duplicated such that there is a total of 25 different tRNA species in A. nidulans mtDNA. The two duplicated A. nidulans tRNAs ($tRNA^{asn}$ and $tRNA^{cys}$) are not the ones duplicated in N. crassa ($tRNA^{fmet}$ and $tRNA^{met}$). The COI gene in A. nidulans contains three introns (all Group I) none of which are at sites equivalent to any of those of the introns in the S. cerevisiae or N. crassa cyt b genes. The second and third introns contain ORFs, and could code for maturases. The A. nidulans cyt b gene contains one intron (Group I) which is in the same position as the third intron in the long form of the cyt b gene of S. cerevisiae. The intron contains an ORF that could code for a maturase. The large (23S) rRNA gene of A. nidulans contains one intron (Group I) that is located at the same site as those of the introns in the corresponding genes of S. cerevisiae and N. crassa. The intron contains an ORF which is homologous to the

ORF in the intron of the 24S rRNA gene of *N. crassa*. As in *N. crassa*, the functional ATPase 9 subunit has been shown to be encoded in the nucleus (Turner et al, 1979), so that the presence of a mt ATPase 9 gene was unexpected. It is not known whether the mt ATPase 9 gene is completely silent or not. The *A. nidulans* mt genome is tightly packed with only about 18% (6 kb) taken up by non-coding intergenic regions.

The 19 kb mtDNA of the fission yeast *S. pombe* has been completely sequenced (Lang et al, 1983, 1985; Lang, 1984). The mt genome contains the ubiquitous mt polypeptides COI, COII, COIII, ATPase 6, ATPase 8, ATPase 9, and cyt b. Only one URF has been identified and it is not homologous to any of the mammalian mt URFs. A total of 25 tRNAs, as well as the small (14S) and large (19S) rRNA genes have been identified. The large (19S) rRNA gene contains no introns. The *S. pombe* COI gene contains two introns (both Group I) which contain ORFs that could code for maturases. The second intron is at the same site as that of the third intron in the COI gene of *A. nidulans*. The *S. pombe* cyt b gene contains one intron (Group II) that has an ORF that could code for a maturase. One strain of *S. pombe* has a continuous cyt b gene, although its COI gene is mosaic, and has the smallest known mt genome among fungi (Zimmer et al, 1984; Trinkl et al, 1985).

Information concerning mt genome organization of fungi outside of the Ascomycotina is limited. A restriction map has been published for the 70 kb linear mtDNA of the slime mold Physarum polycephalum (Kwano et al, 1982). Among Basidiomycetes, restriction enzyme patterns have allowed estimation of mt genome sizes for Ustilago cynodontis (76 kb; Mery-Drugeon et al, 1981), Schizophyllum commune (50 kb; Specht et al, 1983), and Agaricus species (98-176 kb; Hintz et al, 1985). There appears to be significant sequence reiteration in Agaricus mtDNA. In addition, gene maps based on heterologous hybridization with petite gene probes, have been published for the 43.3 kb mtDNA of Coprinus cinereus and the 91.1 kb mtDNA of Coprinus stercorarius (Weber et al, 1986).

Among Mastigomycotina, restriction enzyme maps have been published for Allomyces macrogynus (Borkhardt and Delius, 1983), Phytophthora infestans (Klimczak and Prell, 1984), and Achlya species (Hudspeth et al, 1983; Boyd et al, 1984; Shumard et al, 1986). The small and large rRNA genes have been mapped on Allomyces mtDNA and it appears that the large rRNA gene contains an intron. A gene map has been constructed for Achlya mtDNA by heterologous hybridization with petite gene probes. Further comparative discussion of mt genomes of zoosporic fungi will arise from the data reported in this thesis and will be dealt with in the Discussion.

4. Conclusions regarding mt genome organization

Comparisons of the mt genomes from a wide variety of organisms allow the following generalizations.

1) Across the major groups of organisms (protozoans, fungi, plants, metazoans) mtDNAs are highly diverse in size, ranging from the 16 kb mtDNA of mammals, to the large 2500 kb mtDNA of some plants. Among related organisms, mtDNAs may be similar in size, such as in metazoans (15-19 kb), or highly variable in size, such as in fungi (19-115 kb) and plants (200-2500 kb). The larger size of fungal and plant mtDNAs is due to the presence of non-coding intergenic regions, introns, additional coding functions, promiscuous chloroplast DNA, and sequence reiteration.

2) mtDNAs encode a small number of ubiquitous polypeptides - COI, COII, COIII, cyt b, ATPase 6, ATPase 8, and depending on the organism, ATPase 9. In addition, mtDNAs encode small and large rRNAs and 22 to 28 tRNAs (protozoans may encode a reduced number of tRNAs). Most mtDNAs also encode some subunits of the NADH dehydrogenase complex - URF1, URF2, URF3, URF4, URF4L, and URF5 in mammalian mtDNA. The presence of some of these URFs in many mtDNAs (e.g. the mtDNAs of protozoa, green algae, filamentous fungi, amphibians, and insects contain URF5), indicates that their presence is the rule, and that their absence, as in yeast, is the exception.

3) mtDNAs encode some products in one organism but not in others. Yeast and plant mtDNAs encode ATPase 9, whereas metazoan mtDNA does not. Protozoan, green algae, filamentous fungi, and metazoan mtDNAs encode some subunits of the NADH dehydrogenese complex, whereas yeast mtDNAs do not. Plant mtDNAs encode the F1-ATPase subunit alpha, whereas other mtDNAs do not.

4) Fungal and plant mtDNAs contain mosaic genes, whereas metazoan mtDNAs do not. In fungi, the COI, cyt b, and large rRNA genes are preferred candidates for intron insertion. Fungal introns can be classified into two families (Group I and Group II) based on similarities in secondary structure. Introns may or may not contain ORFs. Some fungal intronic ORFs code for maturases. Some introns are self-splicing, such as the large rRNA gene intron, and intron aI5-gamma of the COI gene of S. cerevisiae.

5) Across the major groups of organisms (protozoans, fungi, plants, metazoans) mt gene organization is highly diverse. Among closely related organisms, mt gene organization may be conserved, as in vertebrates, or it may be highly variable, as in fungi and plants.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Growth of organism

The organism used in this study, *Achlya klebsiana*, was obtained from Dr. H.B. LeJohn, University of Manitoba, Winnipeg. *A. klebsiana*, a homothallic strain, is a zoosporic fungi (Mastigomycotina) belonging to the group Oomycetes.

1.1 Growth media

G2Y- 5.0 g yeast extract, 0.5 mL each of 100 mM $CaCl_2$ and 100 mM $MgCl_2$, per L of distilled water.

PYG- 3.0 g dextrose, 1.0 g yeast extract, 1.0 g peptone, per L of distilled water.

1.2 Stock cultures

Achlya was maintained as mats of sporulating mycelium in Petri plates containing 30-40 mL of G2Y medium at room temperature. Subculturing was done by lifting out 3 mats of mycelium with a flame-sterilized inoculating needle and aseptically placing them in a 1 L flask containing 500 mL of G2Y medium. The flask was shaken vigorously for about 30 sec to release spores, then the mycelium lifted out with a sterile needle. Thirty to 40 mL of spore suspension was then poured aseptically into Petri plates. Thick mats of sporulating mycelium developed in about one week. Subculturing was done every 4-8 weeks.

1.3 Preparation of a large-scale spore suspension

A spore suspension in 500 mL of G2Y medium was prepared as described above, and 5 mL aliquots were inoculated into Roux bottles containing 80 mL of G2Y medium. The Roux bottles were incubated horizontally at room temperature for 2-3 days to allow sporulation to occur. Roux bottles were shaken vigorously to suspend spores and the contents filtered through a sterile filtering apparatus consisting of one layer of cotton gauze secured over the bottom of a Millipore filter holder which was inserted into the mouth of a 2800 mL Fernbach flask. The contents of 18-21 Roux bottles yielded a large-scale spore suspension of 1400-1700 mL.

1.4 Preparation and harvesting of a large culture

Equal aliquots from one large-scale spore suspension were used to inoculate 9-11 2 L shaker flasks, each containing 500 mL of PYG medium. The flasks were incubated at 28°C on a platform rotary shaker at 150-170 rpm for 8-12 h.

The mycelium was harvested by suction filtering the contents of the flasks onto Whatman #1 filter paper. The mycelial mat obtained was washed twice with distilled water and then once with 100-200 mL of isolation medium (see below).

2. Isolation of mtDNA

Mitochondria were isolated by the flotation gradient method (Lizardi and Luck, 1971; Lambowitz, 1979), and the mtDNA was isolated from mitochondria by preparation of ribonucleoprotein (RNP) pellets (Lambowitz and Luck, 1976). This was followed by phenol extraction, RNase treatment (Manella and Lambowitz, 1978), and ethanol precipitation. These procedures are described below.

2.1 Buffers, chemicals, materials

Water- The water used for all solutions was distilled and deionized.

Acid-washed sand- Medium grain sand was soaked in concentrated HCl overnight, washed thoroughly with distilled water, washed with 20 mM EDTA, washed with distilled-deionized water, dried in an oven, and stored at 4°C.

Isolation medium- 15% sucrose in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA.

Sucrose gradient solutions- 60%, 55%, and 44% sucrose solutions in 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA.

HKCTD- 500 mM KCl, 50 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.6, 5 mM DTT, made up fresh in acid-washed glassware.

Nonidet- 20% Nonidet P-40 in HKCTD.

Sucrose cushion- 1.85 M sucrose in HKCTD.

NSET- 100 mM NaCl, 100 mM Tris-HCl, pH 8.2, 2 mM EDTA.

HNET- 100 mM NaCl, 100 mM Tris-HCl, pH 8.2, 1 mM EDTA.

Phenol- Freshly distilled phenol was saturated with HNET and stored frozen in a foil-covered container.

HNET dialysis buffer- 150 mM NaCl, 100 mM Tris-HCl, pH 8.0, 0.625 mM EDTA.

RNase- 2.0 mg/mL RNase A in HNET was stored frozen. On the day of use, 0.2 mL of RNase A was mixed with 5 uL of RNase T1 and incubated at 80°C for 10 min.

TE buffer- 10 mM Tris-HCl, pH 7.6, 1mM EDTA.

2.2 Disruption of cells and isolation of crude mt pellets

Disruption of cells was carried out in a cold room at 10°C.

Approximately 20-40 g of cells at a time were placed in a mortar (700-800 mL) that had been rinsed with a small volume of isolation medium. Acid-washed sand approximately equal to cell mass was added. Isolation medium was added slowly and mixed with a pestle until the sand-cell mixture was a paste consistency and would just adhere to the sides of the mortar. Grinding was continued for a further 2-5 min until all major cell clumps were dispersed. Approximately 100-250 mL of

isolation medium was added to the mortar, the contents mixed, and the sand allowed to settle to the bottom. The suspension was centrifuged for 10 min at 3,000 rpm in a Sorvall SS-34 rotor at 4°C. The supernatants were decanted, leaving behind the crude mt pellets.

2.3 Purification of mitochondria

Ten mL of 60% sucrose solution was added to one tube and the crude mt pellet suspended with a pre-chilled glass pestle with care taken not to suspend residual sand at the bottom of the pellet. This suspension was used to suspend the pellets in the other tubes until all the pellets were suspended in one tube, with the clumps being further suspended by gentle vortexing. The suspension was made up to 24 mL with 60% sucrose and 4 mL aliquots were pipetted into six 14 X 89 mm polyallomer tubes (Beckman). Each aliquot was overlayered with 4 mL of 55% sucrose solution which was then overlayered with 44% sucrose solution to within about 3 mm of the top of the tube. The tubes were placed into pre-chilled tube holders and centrifuged for 90 min at 40,000 rpm, or for 120 min at 37,000 rpm, in a SW 41 rotor (Beckman) at 4°C. After centrifugation, the mt band at the 44%/55% sucrose interface was collected with a Pasteur pipette, diluted 1:3 with HKCTD, mixed by inversion, and centrifuged for 30 min at 13,000 rpm in a Sorvall SS-34 rotor at 4°C to obtain the final mt pellet.

2.4 Extraction of mtDNA from mitochondria

The final mt pellet was resuspended in 3.8 mL of HKCTD by means of a cold glass pestle. With gentle swirling, 0.2 mL of warm 20% Nonidet was added to the suspension. Clearing occurred almost immediately. The lysed mitochondria were layered on 5.5 mL of sucrose cushion in a 16 X 79 mm polyallomar centrifuge tube (Beckman) and centrifugation carried out for at least 18 h at 50,000 rpm in a 50 Ti rotor (Beckman), or for at least 16 h at 55,000 rpm in an 80 Ti rotor (Beckman), at 4°C.

After centrifugation, the top layer plus a 1 cm depth of sucrose cushion was aspirated off and the remainder was overlayered with cold ultrapure water. The water plus another 1 cm depth of sucrose cushion was aspirated off, cold water overlayered on the remainder, and the rest of the aspiration-overlayering procedure continued until all the sucrose cushion was gone, leaving behind the RNP pellet. The surface of the RNP pellet was rinsed once with cold water and then resuspended in 2 mL of warm NSET. The suspension was transferred to a 15 mL polypropylene centrifuge tube and an equal volume of buffered phenol added. The tube was gently swirled until the solution was totally cloudy, then allowed to stand 10 min with two swirlings in between. It was then centrifuged for 10 min at 10,000 rpm in a Sorvall SS-34 rotor at room temperature.

The top aqueous layer was collected and extracted with buffered phenol as before and the top aqueous layer was placed in a suitable length of dialysis tubing and dialysed overnight against 4 L of HNET dialysis buffer.

The dialysate was collected, 25 uL of RNase solution was added per mL of dialysate, and the mixture was incubated at 37°C for 30 min. Two vol of 95% ethanol was added, the solution mixed and held at -60°C for at least 30 min, or -20°C overnight, to precipitate the mtDNA. The DNA was collected by centrifugation for 10 min at 10,000 rpm in a Sorvall SS-34 rotor at 0°C. The ethanol was decanted and the pellet dried in a desiccator under vacuum. The DNA pellet was dissolved in 50-100 uL of TE buffer and stored at 4°C.

The concentration of the DNA solution was determined by measuring the absorbance at 260 nm, and the purity of the solution was determined by further measuring the absorbance at 280 nm and calculating the A_{260}/A_{280} ratio (Maniatis et al, 1982). DNA with a ratio of 1.8 to 2.0 was considered relatively pure.

3. Restriction enzyme digestion and gel electrophoresis of mtDNA

Restriction enzymes were purchased from Bethesda Research Laboratories or Boehringer Mannhiem Canada.

Digestions were carried out in 1.5 mL micro-centrifuge tubes with 0.5-1.0 ug of Achlya mtDNA and 5-10 units of enzyme, in buffers recommended by the suppliers. Multiple enzyme digestions were carried out simultaneously using intermediate assay conditions, or sequentially so that different recommended assay conditions for each enzyme could be followed. Incubations were carried out for 1-3 h. Reactions were terminated by addition of 0.1 vol of a solution containing 25% Ficoll, 0.25% bromophenol blue, and 100 mM EDTA.

Electrophoresis in agarose gels (0.4%-1.0%) was done in 15 X 16 cm or 25 X 20 cm slabs run as horizontal submarine gels, and was most often carried out for 12-20 h at 35 V for the 15 cm gels, or 50-60 V for the larger gels. Electrophoresis in polyacrylamide gels (4.0%-8.0%) was done in 17 X 16 cm gels in a vertical apparatus, and was carried out at 70-90 V for 4-6 h, or at 35 V for 12-16 h. Electrophoresis buffer for both types of gels was 0.089 M Tris base, 0.089 M boric acid, 2.5 mM EDTA, pH 8.0.

Gels were stained for 30 min by submerging them in electrophoresis buffer containing 1 ug/mL of ethidium bromide.

Photographs of gels were taken by placing the gel on an ultraviolet transparent tray on a black background and exposing Polaroid type 667 film through a Wratten 22A filter, with illumination being provided by one or two Mineralight lamps (Ultraviolet Products Inc., San Gabriel, USA).

Restriction digest fragments of bacteriophage lambda DNA and/or plasmid pBR322 DNA were used for molecular size standards as the complete nucleotide sequences of these DNAs have been determined (Sanger et al, 1982; Sutcliffe, 1979). Fragment migration distances were measured on the photographs from the the bottom of the loading wells to the middle of the fragment band. Fragment sizes were estimated by reference to a standard curve constructed from the lambda DNA and/or pBR322 DNA restriction digestion fragments by assuming mobility in agarose or polyacrylamide gels to be inversely proportional to the $\log_{10}$ of fragment size. All fragment sizes are expressed in either kilobase pairs (kb) or base pairs (bp).

4. Cloning of A. klebsiana mtDNA

4.1 Bacterial host strains

All bacteria used as hosts for plasmids were derived from *Escherichia coli* K12:

E. coli HB101 (Boyer and Roulland-Dussoix, 1969) was obtained from Dr. P. Loewen, University of Manitoba. Genotype: *F*$^-$, *hsdS20*(r_B^-,m_B^-), *supE44*, *ara-14*, *galK-12*, *lacY1*, *proA2*, *rpsL20*, *str*^{r}, *xyl-5*, *mtl-1*, *leu*$^-$, *lambda*$^-$.

E. coli JM103 (Messing et al, 1981) was obtained from Dr. H. Duckworth, University of Manitoba. Genotype: *lacpro*, *thi*, *strA*, *supE*, *endA*, *sbcB15*, *hsdR4*, *F'traD36*, *lacI*^{q}, *Z*Δ*M15*.

4.2 Cloning vectors

Plasmid *pBR322* (Bolivar et al, 1977) was obtained from Dr. P. Loewen. The host was *E. coli* HB101. Plasmid *pBR322* is 4362 bp (Sutcliffe, 1979) and contains genes whose products confer resistance to ampicillin and tetracycline upon the host cell.

Plasmid *pUC9* (Vieira and Messing, 1982) was obtained from Dr. H. Duckworth. The host was *E. coli* JM103. Plasmid *pUC9* is approximately 2700 bp and contains part of the beta-galactosidase gene from *E. coli* and a gene whose product confers resistance to ampicillin upon the host cell.

4.3 Media and reagents

LB medium- 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, per L of distilled water. For plates 1.5% agar was added.

2YT medium- 16.0 g tryptone, 10.0 g yeast extract, 5.0 g NaCl, per L of distilled water. For plates 1.5% agar was added.

Glucose minimal medium- 6.0 g $NaHPO_4$, 0.5 g NaCl, 1.0 g NH_4Cl, per L of distilled water, pH adjusted to 7.4 with NaOH, supplemented after autoclaving with 1.0 mL of 100 mM $CaCl_2$, 1.0 mL of 1 M $MgSO_4$, 10 mL of 30% glucose, and 0.5 mL of thiamine. For plates 1.5% agar was added.

Ampicillin- A 15 mg/mL stock solution in water was stored at -20°C. For plates containing approximately 30 mL of medium, 0.1 mL aliquots of stock solution were spread on plates just before use so that the final concentration was approximately 50 ug/mL.

Tetracycline- A 4.5 mg/mL stock solution in 50% ethanol was stored at -20°C. For plates containing approximately 30 mL of medium, 0.1 mL aliquots of stock solution were spread on plates just before use so that the final concentration was approximately 15 ug/mL.

Chloramphenicol- A 34 mg/mL stock solution in 100% ethanol was stored at -20°C. Chloramphenicol was added to medium at a final concentration of 170 ug/mL (a 1:200 dilution of stock solution) for plasmid amplification.

X-gal- A 20 mg/mL stock solution in dimethylformamide was stored at 4°C in the dark. X-gal was added to molten medium at 55°C to a final concentration of 40 ug/mL (a 1:500 dilution of stock solution) before pouring plates.

IPTG- A 100 mM stock solution in water was stored at -20°C. IPTG was added to molten medium at 55°C to a final concentration of 0.2 mM (a 1:500 dilution of stock solution) before pouring plates.

10X ligase buffer- 300 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, stored at -20°C.

10X calf intestinal alkaline phosphatase (CIAP) incubation buffer- 500 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, stored at 4°C.

10X TNE buffer- 500 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, stored at 4°C.

4.4 Plasmid isolation and restriction analysis of plasmid DNA

The method for small scale plasmid isolations was adapted from the alkaline extraction method as described below (Birnboim, 1983).

For isolation of plasmids harbored in *E. coli* HB101 or JM103, 10 mL cultures in LB medium containing ampicillin were grown overnight at 37°C with shaking. The cells were collected by centrifugation at 5,000 rpm for 5 min in a Sorvall SS-34 rotor at 4°C. Each pellet was resuspended in 0.5 mL of glucose buffer containing 50 mM glucose, 20 mM EDTA, and 25 mM Tris-HCl, pH 8.0. A further 0.5 mL of glucose buffer containing 2 mg/mL of lysozyme was added to each cell suspension. The suspensions were mixed and held on ice for 10 min. Two mL of akaline-SDS solution containing 0.2 N NaOH and 1% SDS was added and the suspensions mixed and held on ice for 20 min. Next, 1.5 mL of high salt solution containing 3 M potassium acetate and 1.8 M formic acid was added and the suspensions mixed and held on ice for 30 min. The suspensions were then centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 4°C. The supernatants were transferred to new tubes and 2 vol of 95% ethanol was added. The tubes were mixed thoroughly, held at -20°C for 20 min, then centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 0°C.

The pellets were suspended in 100-300 uL of TE buffer, and the DNA stored at 4°C.

Large scale plasmid isolations were done with chloramphenicol amplified cultures (Clewell, 1972) by a preparative alkaline extraction method as described below (Birnboim, 1983).

For isolation of plasmids harbored in E. coli HB101, 5 mL of a 10 mL overnight culture in LB medium containing ampicillin was used as the inoculum for 500 mL of LB medium containing ampicillin in a 2 L shaker flask. When E. coli JM103 was the host, 500 mL of 2YT medium was used. The flasks were incubated at 37°C on a platform rotary shaker at medium-high speed. When the $O.D._{600}$ reached 0.6-0.9, chloramphenicol was added to a final concentration of 170 ug/mL and the incubation continued overnight (Clewell, 1972; Maniatis et al, 1982).

Cells were collected by centrifugation at 5,000 rpm for 10 min in a Sorvall GSA rotor at 4°C. The pellets were consolidated in 30-40 mL of water and centrifuged at 5,000 rpm for 10 min in Sorvall SS-34 rotor at 4°C. The cell pellet was then suspended in 1 mL of glucose buffer, after which a further 9 mL of glucose buffer containing 10 mg of lysozyme was added. The suspension was mixed and held on ice for 30 min. Twenty mL of alkaline-SDS was added and the suspension was mixed and held on ice for 15 min. Fifteen mL of high-salt

solution was added and the suspension mixed and held on ice for 30 min. The suspension was then centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 4°C. The supernatant was transferred to a new tube and 2 vol of 95% ethanol was added and the tube mixed and held at -20°C for 20 min. The nucleic acids were pelleted by centrifugation at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 0°C. The pellet was dissolved in 5 mL of 0.1 M sodium acetate, 0.05 M MOPS (morpholino-propanesulfonic acid) solution, pH 8.0, then precipitated with ethanol and collected by centrifugation as before. The nucleic acid pellet was dissolved in 2 mL of water and then mixed with an equal volume of 5 M LiCl, 0.05 M MOPS solution, pH 8.0, and held on ice for 15 min. The solution was then centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 0°C. The supernatant was collected and heated at 60°C for 10 min and if a precipitate formed it was removed by centrifugation. Nucleic acids were precipitated twice with ethanol and the final pellet dissolved in 2 mL of TE buffer. Twenty uL of RNase solution was added (see Materials and Methods section 2.1) and the mixture incubated at 37°C for 30 min. After incubation, 40 uL of 10% SDS and 2 mL of acetate-MOPS solutions were added and the contents mixed. Next, 4 mL of isopropanol was added dropwise with mixing and the solution allowed to stand at room

temperature for 15 min. The plasmid DNA was collected by centrifugation at 10,000 rpm for 10 min in a Sorvall SS-34 rotor at 20°C. The DNA pellet was dissolved in 2 mL of acetate-MOPS, precipitated with ethanol and collected by centrifugation as before, and then dried in a desiccator under vacuum. The pellet was dissolved in 0.5-2.0 mL of TE buffer and stored at 4°C.

Restriction enzyme digestion and gel electrophoresis of plasmid DNA was as described for Achlya mtDNA except that agarose gels of 0.8%-2.0% were used (Materials and Methods section 3).

4.5 Preparation of competent host cells

Host strains were made competent for transformation by treatment with $CaCl_2$ as described below (Cohen et al, 1972).

For E. coli HB101, 1 mL of a 10 mL overnight culture in LB medium (with ampicillin) was used as the inoculum for 100 mL of LB medium containing ampicillin in a 500 mL shaker flask. For E. coli JM103, 100 mL of 2YT medium containing ampicillin was inoculated with 1 mL of a 10 mL overnight culture. The flask was incubated at 37°C for 2-3 h with vigorous shaking. The culture was placed on ice for 20 min, then 30 mL of it decanted into a 30 mL sterile Corex tube and centrifuged at 5,000 rpm for 5 min in a Sorvall SS-34 rotor at 4°C. The cell pellet was then suspended in 5 mL of cold 100 mM $CaCl_2$ and the suspension kept on ice for

20 min. The cells were collected by centrifugation as before, the cells resuspended in 2 mL of cold 100 mM $CaCl_2$, kept on ice overnight, and used for transformation that day.

4.6 Transformation of host cells with plasmid DNA

Transformation of competent host cells was according to Maniatis et al, 1982.

Aliquots (0.2 mL) of competent host cells were placed in prechilled 1.5 mL microcentrifuge tubes, the plasmid DNA (50-100 ng) added, the contents mixed, and the tube placed on ice for 30-60 min. The tube was then placed at 37°C for 5 min, followed by the addition of 1 mL of LB medium and further incubation at 37°C for 45-60 min. The suspension was then centrifuged at full speed for 1 min in a microcentrifuge, the supernatant decanted, and the cells resuspended in 1 mL of fresh LB medium. Aliquots of cells (100 uL per plate) were used for isolation of appropriate transformants as described below (Materials and Methods sections 4.7 and 4.8).

4.7 Cloning into pBR322

One ug of Achlya mtDNA was digested to completion with BclI. BclI recognizes the sequence 5'-T↓GATCA-3', and generates DNA fragments with 5' cohesive ends (Bingham et al, 1978). Two hundred and thirty ng of pBR322 was digested to completion with BamHI. BamHI recognizes the sequence 5'-G↓GATCC-3', and generates DNA fragments with 5' cohesive ends identical to the 5' ends generated by BclI (Wilson and Young, 1975; Roberts et al, 1977). The two digested DNA mixtures were pooled, brought up to 100 uL with TE buffer, and extracted with an equal volume of phenol. The top aqueous layer was collected and the DNA precipitated by adding 2 vol of 95% ethanol and then holding at -60°C for 15 min. The DNA was collected by centrifugation in a microcentrifuge at full speed for 10 min and the pellet suspended in 43.5 uL of 10 mM Tris-HCl, pH 7.6. Five uL of 10X ligation buffer, 0.5 uL of 100 mM ATP, and 1 uL of T4 DNA ligase (1 unit/uL), were added to the DNA mixture, bringing the total volume to 50 uL. Ligation was carried out in a 1.5 mL microcentrifuge tube at 10°C for 14 h.

A 10 uL aliquot of the ligation mixture was used to transform E. coli HB101 as described above (Materials and Methods section 4.6). Aliquots of cells (100 uL) were spread on LB plates containing ampicillin and incubated upside down overnight at 37°C.

Transformants that grew overnight were transferred with sterile toothpicks to LB plates containing ampicillin and to LB plates containing tetracycline and incubated overnight as before. The ampicillin resistant cells that were tetracycline sensitive were transferred to fresh LB plates containing ampicillin, incubated overnight as before, and then stored at 4°C for up to one month before the colonies were transferred to a fresh LB-ampicillin plate. Small and/or large volume plasmid preparations were done on these colonies as described above (Materials and Methods section 4.4).

4.8 Cloning into pUC9

Two hundred ng of Achlya mtDNA was digested to completion with EcoRI. EcoRI recognizes the sequence 5'-G↓AATTC-3', and generates DNA fragments with 5' cohesive ends (Hedgpeth et al, 1972). The solution was brought up to 100 uL by addition of TE buffer, extracted with an equal volume of phenol, precipitated with ethanol, and the pellet dissolved in 10 uL of 10 mM Tris-HCL, pH 7.6.

Five ug of pUC9 was digested to completion with EcoRI, phenol extracted, precipitated with ethanol, and the pellet dissolved in 44 uL of 5 mM Tris-HCl, pH 8.0. Five uL of 10X CIAP buffer and 1 uL of CIAP (0.1 unit/uL) were added to the DNA solution, and incubation was carried out for 30 min at 37°C.

Inactivation of the phosphatase was as follows (Maniatis et al, 1982). Ten uL of 10X TNE buffer, 35 uL of H_2O, and 5 uL of 10% SDS, were added to the DNA mixture and incubation carried out at 68°C for 15 min. The mixture was then extracted once with phenol:chloroform (1:1 v/v) and twice with chloroform. It was then precipitated with ethanol and the pellet dissolved in 50 uL of TE buffer. The concentration of the phosphatase-treated pUC9 was 35 ng/uL as measured by absorbance at 260 nm.

To 10 uL Achlya mtDNA was added 10 uL of the phosphatase-treated pUC9 (350 ng), 23.5 uL of 10 mM Tris-HCl, pH 7.6, 5 uL of 10X ligation buffer, 0.5 uL of 100 mM ATP, and 1 unit of T4 DNA ligase (1 unit/uL). The components were mixed in a 1.5 mL microcentrifuge tube and incubated at 10°C for 14 h.

A 10 uL aliquot of the ligation mixture was used to transform E. coli JM103 as described above (Materials and Methods section 4.6). Aliquots of the cells (100 uL) were spread on 2YT plates containing X-gal, IPTG, and ampicillin, and incubated upside down overnight at 37°C. Transformants that grew as white colonies were transferred to fresh 2YT plates containing ampicillin. Within 3-4 weeks, transformants were transferred to glucose minimal plates and stored at 4°C.

Small and/or large volume plasmid preparations were done on these colonies as described above (Materials and Methods section 4.4).

5. DNA-DNA hybridization

5.1 Buffers and solutions

Denaturing solution- 0.5 M NaOH, 1.5 M NaCl.

Neutralizing solutions- A) 1.0 M Tris-Hcl, pH 8.0, 1.5 M NaCl.

B) 1.0 M ammonium acetate, 0.02 M NaOH.

10X nick translation buffer- 500 mM Tris-HCl, pH 7.2, 100 mM $MgCl_2$, 1 mM DTT, 500 ug/mL BSA, stored at -20°C.

1X SSC- 0.15 M NaCl, 0.015 M sodium citrate.

1X Denhardt's solution- 0.2% Ficoll, 0.2% BSA, and 0.2% polyvinylpyrrolidone in distilled water, stored at -20°C.

Hybridization buffer- 6X SSC, 0.5% SDS, 5X Denhardt's solution, 100 ug/mL denatured salmon sperm DNA, stored at 4°C.

Wash solutions- #1- 2X SSC, 0.5% SDS.

#2- 2X SSC, 0.1% SDS.

#3- 0.1X SSC, 0.5% SDS.

5.2 Immobilization of DNA on nitrocellulose filters

Restriction digests of DNA were immobilized on nitrocellulose filters after gel electrophoresis by modified Southern blotting (Southern, 1979), or by the bidirectional transfer method (Smith and Summers, 1980), as described below.

Gels were trimmed and soaked in at least 2 vol of denaturing solution for 1 h. The gel was then rinsed with distilled water and soaked in either neutralizing solution A or B for 1 h.

Nitrocellulose filters were cut to the same size as the gel, floated on the surface of 2X SSC or neutralizing solution B until completely wet from beneath, and then immersed completely for 2-3 min.

For Southern blotting, a paper wick of Whatman 3MM paper was placed on a glass plate suspended over an enamelled baking dish, and the dish filled with 10X SSC. The wick was completely wetted with 10X SSC, the neutralized gel placed on top of the wick, and the wetted nitrocellulose filter placed on top of the gel, with care being taken not to leave trapped air bubbles between layers. Two pieces of Whatman 3MM paper, cut to the same size as the gel, were wetted in 2X SSC and placed on top of the nitrocellulose filter. The area of the wick around the gel was covered by Saran Wrap, a 2 inch high stack of paper towels placed on the covered filter, and a weight (approximately 500 g)

placed on top. Transfer was allowed to proceed for 12-24 h.

For bidirectional transfer, a piece of nitrocellulose saturated with neutralizing solution B was placed on three pieces of similar sized Whatman 3MM paper that had been saturated with the neutralizing solution. The neutralized gel was placed on top of the nitrocellulose filter, and a second piece of cut nitrocellulose placed on top of the gel. Three pieces of Whatman 3MM paper that had been saturated with neutralizing solution B were placed on top of the second nitrocellulose filter. A stack of paper towels was placed on the covered filter, weighted on top, and the transfer allowed to proceed as for Southern blotting.

After transfer, nitrocellulose filters were rinsed in 6X SSC at room temperature for 5 min, air dried on a piece of Whatman 3MM paper for an hour, then baked at 80 ^{o}C for 2 h between two pieces of 3MM paper. Baked filters, together with the 3MM paper, were wrapped loosely in aluminium foil and stored in a desiccator under vacuum.

5.3 Radioactive labelling of DNA, hybridization, and autoradiography

DNA was radioactively labelled by nick translation as described below (Rigby et al, 1977).

One ug of DNA, 5 uL of 10X nick translation buffer, 1 uL each of 1 mM solutions of dATP, dGTP, and dTTP, 5 uL of [alpha-^{32}P]dCTP (63 pmoles at 80 uCi/pmole), 1 uL of a 0.1 ug/mL DNase I solution (in nick translation buffer containing 50% glycerol), 1 uL of *E. coli* DNA polymerase I (5 units/uL), and ultrapure water to bring the total volume up to 50 uL, were mixed in a 1.5 mL microcentrifuge tube and incubated at 16 ^{o}C for 60-90 min. The reaction was terminated by the addition of 2 uL of 0.5 M EDTA (final concentration of EDTA was 20 mM).

Nick translated DNA was separated from unincorporated nucleotides by chromatography on a small column of Sephadex G-50 as described below (Maniatis et al, 1982). A plastic disposable 5 mL pipette with a siliconized glass wool plug in the tip was filled with 5 mL of Sephadex G-50 that had been equilibriated in TE buffer. The nick translation reaction mixture was loaded on to the top of the column and allowed to enter the Sephadex. The labelled DNA was eluted with TE buffer and collected after following the leading peak of radioactivity with a Geiger counter.

Prehybridization, hybridization, and washing of nitrocellulose filters were as described below (Maniatis et al, 1982).

Prehybridization was carried out at 65°C for 2-4 h in a flat bottomed pan containing 100-200 mL of hybridization fluid. Hybridization was carried out at 65°C for 12-48 h in heat-sealed plastic bags containing the denatured labelled DNA and 10-20 mL of hybridization fluid. Labelled DNA was denatured by boiling for 10 min. Hybridized filters were washed in wash solution #1 for 5 min at room temperature, followed by wash solution #2 for 15 min at room temperature, followed by wash solution #3 at 65°C for 1-3 h.

Filters were air dried for 1 h, then wrapped in Saran Wrap and autoradiographed for 12-72 h on Kodak X-Omat RP film in Kodak X-Omatic cassettes with intensifying screens. Autoradiography was carried out either at room temperature or at -70°C.

RESULTS

RESULTS

1. Isolation of the mtDNA

Isolation of the mtDNA from *A. klebsiana* was done in three basic steps: 1) isolation of mitochondria from ruptured hyphae by the flotation gradient method, 2) preparation of ribonucleoprotein (RNP) pellets from lysed mitochondria, and 3) phenol extraction and RNase treatment of the RNP pellets, followed by ethanol precipitation of the mtDNA (Materials and Methods section 2). The above procedures, which were initially developed for the isolation of intact mt rRNA and mtDNA from the filamentous ascomycete *Neurospora crassa*, involved several steps important for consistent isolation ot mtDNA. Excessive grinding of the hyphae with sand inevitably led to low numbers of mitochondria being recovered from the sucrose gradients. It was found that grinding for 2-5 min resulted in the best recovery of mitochondria. Inclusion of $CaCl_2$ in the HKCTD was assumed to be critical as it was found that in *N. crassa* the Ca^{2+} supressed the nuclease(s) released during lysis of the mitochondria (Lambowitz and Luck, 1976; Lambowitz, 1979). An additional protease-phenol extraction step after the RNase treatment (Manella and Lambowitz, 1978) was found not to be necessary in isolating mtDNA suitable for restriction digestion or increasing yield, and so was

not included. Applied to A. klebsiana , it was found that these procedures consistently resulted in the isolation of 10-20 ug of mtDNA from 40-80 g wet weight of mycelium. This allowed only a small number of restriction digests to be done with any one mtDNA preparation. To determine if greater yields of mtDNA could be obtained from the same amount of starting material, two other methods for isolating mtDNA from filamentous fungi were attempted (Hudspeth et al, 1983; Garber and Yoder, 1983). Both methods involve isolating the mtDNA as a distinct band from CsCl-bisbenzimide gradients. They were unsucessful, however, when applied to A. klebsiana; the mtDNA band was either absent or so faint as to be irretrievable. Hence, the method used here, which consistently resulted in isolation of mtDNA suitable for restriction digestion, was the method of choice. It may be that in A. klebsiana the percentage of total DNA that is mt is small. It is also noted that the percentage of mtDNA isolated as intact circles was quite small. However, the mtDNA was sufficiently intact to allow restriction mapping with all the enzymes used, even though the large restriction fragments may have been present in sub-molar amounts.

2. Restriction mapping of the A. klebsiana mt genome

2.1 Nomenclature of restriction fragments

Each restriction fragment will be referred to by the full name of the restriction enzyme(s) that generated it, followed by a number indicating the size rank of the fragment, from largest to smallest. In the case of a double band (2 molar amount of DNA), a small letter, either a or b, will follow the number, indicating that only that fragment, representing one half of the double band family, is being referred to.

2.2 Approximate size of the mt genome

Typical agarose gel patterns of the fragments for single and multiple digestions of the mtDNA are shown in the Results Appendix (Figs. 22-27). A prominent feature of these digests is that in some cases one or more bands appear to be present in greater than molar amounts (Figs. 22-27, white circles). When such bands were cleaved by other enzymes to yield bands which were also obviously double bands, the interpretation was that the original double band consisted of a homogeneous set of sequences in more than molar amount. This was taken to be preliminary evidence for sequence reiteration in the mt genome. This interpretation was confirmed as a consistent picture of mt genome size and arrangement emerged from deductions based on the interpretation. Table 1 lists the molecular sizes (kb) of the fragments generated by single digestions of the

mtDNA with eight restriction enzymes that cleaved from 6-11 times. The mean of the sums of the various single digests was 50.82 kb. Tables 2a and 2b list the molecular sizes (kb) of the fragments generated by double digestions of the mtDNA with various combinations of the eight enzymes listed in Table 1. The mean of the sums of the various double digestions was 49.83 kb. The fragment sizes listed in Tables 1, 2a and 2b were based on the mean sizes of products from a total of 150 single and double digestions.

Since the sizes of large fragments (>10 kb) tend to be overestimated because their sizes are calculated from the least accurate part of the graphs used to determine fragment sizes, the sums of the single digests may be slight overestimations of the actual mt genome size. Conversely, since small fragments (<0.5 kb) were not detected on agarose gels under the standard electrophoretic conditions, the sums of the various double digestions may be slight under-estimations of the actual genome size. Hence, the mean of the sums of the single and double digestions, 50.3 kb $\pm$ 1.5 kb (51.36 kb-48.33 kb = 3 kb; 3/2 = 1.5; largest minus smallest size estimation divided by 2), was taken to be an accurate estimation of the actual mt genome size.

Table 1. Molecular size (kb) of fragments generated by restriction enzyme cleavage of A. klebsiana mtDNA.

Fragment number	Restriction enzyme							
	BclI	BglII	BstEII	ClaI	EcoRI	HincII	HpaI	SstI
1	17.10	11.90	15.80	17.30	13.10	9.99	15.50	18.60
2	8.80	11.38	11.85	11.83	9.65	8.80	11.30	15.20
3	5.45	7.51	6.23	10.65	8.57	7.90	10.00	7.10
4	*4.86	7.04	*5.40	6.33	7.43	*6.15	8.80	*2.86
5	*3.06	6.22	*3.14	2.82	6.70	4.85	2.20	*2.37
6	2.42	2.80		1.82	3.19	2.18	1.61	
7	1.32	1.63			0.93	1.61	1.32	
8		*0.85			*0.87	1.56		
9		*0.20				1.32		
10						0.30		
Sums	50.93	50.58	50.96	50.75	50.44	50.81	50.73	51.36
Mean	50.82							

* Fragments represented in twice molar amount.

Table 2a. Molecular size (kb) of fragments generated by double digestion of A. klebsiana mtDNA with restriction enzymes.

	Restriction enzymes						
Fragment number	BclI x BglII	BclI x BstEII	BclI x HincII	BglII x BstEII	BglII x ClaI	BglII x HincII	BglII x SstI
1	7.45	9.10	7.00	7.60	11.90	7.40	8.80
2	6.50	6.80	6.80	*5.40[a]	9.20	*6.00	8.10
3	5.70	6.30	5.50	4.70	7.50	4.85	7.10
4	*4.50	5.50	3.70	4.40	3.60	2.80	5.70
5	3.90	*3.40	*3.60	2.95	2.90	2.70	5.35
6	*3.04	2.40	2.40	1.90	2.80	2.60	2.40
7	2.90	*1.85	*2.30	1.64	2.60	2.20	2.10
8	2.10	*1.70	2.23	*1.10	2.10	1.85	1.84
9	1.42	*1.45	1.60	*1.00	1.87	1.63	1.63
10	*1.32	1.31	1.55	*0.86	1.63	1.60	*0.90[b]
11	*0.87	*1.18	1.32		1.52	1.56	*0.86
12			1.31		*0.86	1.32	
13			*1.20			1.20	
14			*0.70			1.10	
15						*0.90	
16						*0.86	
Sums	49.63	50.57	49.01	50.71	49.35	48.33	50.74

Only the fragments that were visualized on agarose gels are listed.

* Fragments represented in twice molar amount.

a) Quadruple band consisting of a double fragment and two other non-homologous similar-sized fragments.

b) Quadruple band consisting of two pairs of similar-sized double fragments.

Table 2b. Molecular size (kb) of fragments generated by double digestion of A. klebsiana mtDNA with restriction enzymes.

Fragment number	Restriction enzymes: BstEII x ClaI	BstEII x HincII	BstEII x SstI	ClaI x HincII	ClaI x SstI	EcoRI x HincII	EcoRI x HpaI	HincII x SstI
1	12.20	10.00	11.90	9.70	12.50	5.60	8.82	9.20
2	9.50	8.70	9.25	8.75	7.40	4.91	7.00	7.50
3	5.55	*5.40	7.06	*6.00	6.00	4.00	*5.60	4.90
4	*5.40	4.90	6.16	5.25	5.80	3.80	3.45	*3.60
5	2.60	*2.80	*3.10	2.50	4.65	3.50	3.21	*2.87
6	*2.48	2.18	*2.86	*2.20	*2.42	3.19	*2.86	*2.35[a]
7	2.30	2.00	*2.16	2.07	2.40	*2.70	2.56	2.16
8	*0.70	1.80		1.60	*2.30	2.55	2.20	1.94
9	*0.60	1.60		1.10	*0.60	*2.50	1.95	1.62
10		1.45		0.85		2.16	1.35	1.58
11		1.32		0.75		2.00	0.94	1.33
12						1.59	*0.88	
13						1.55		
14						1.33		
15						0.95		
16						*0.87		
Sums	50.51	50.35	50.61	48.97	49.39	49.27	49.79	50.22
Mean[b]	49.83							

Only the fragments that were visualized on agarose gels are listed.

* Fragments represented in twice molar amount.

a) Triple band consisting of a double fragment and a non-homologous similar-sized fragment.

b) Mean of the sums from both Table 2a and Table 2b.

2.3 Mapping strategy

Fragment orders were deduced from the molecular sizes of fragments generated from single and multiple digests of the mtDNA (Tables 1, 2a, and 2b). Fragment ordering was facilitated by the use of two enzymes, AvaI and PvuII, each of which cleaved the mtDNA at only one site. Codigestion of the mtDNA with AvaI or PvuII, together with one or more of the multisite enzymes, provided information about the overlap of fragments in digests by different restriction enzymes.

The orientation of the fragments cleaved by AvaI or PvuII was determined by comparison of the molecular sizes of fragments generated in double digests with those expected for the different possible orientations. Table 3 lists the fragments cleaved by AvaI and PvuII and the corresponding sub-fragments. Fragment ordering (or confirmation of fragment orders) was also facilitated by codigestions of the mtDNA with PstI, SalI, BamHI, or SstII, together with one of the multisite enzymes, since these enzymes cleaved the mtDNA at only two sites. Similar reasoning to that used for codigestions with the single-site enzymes was used to determine overlap information and orientation of fragments cleaved by these enzymes. Table 4 lists the fragments cleaved by PstI, SalI, BamHI, and SstII, and the corresponding sub-fragments.

Table 3. Cleavage products generated by the single-site enzymes AvaI and PvuII.

Fragment cleaved[a]	Size (kb)	Double digestion cleavage products	
		AvaI	PvuII
BglII 2	11.58	9.70, 1.88	
BglII 4	7.04		4.58, 2.35
HincII 3	8.04		7.55, 0.41[b]
HpaI 1	15.60		13.50, 2.05
EcoRI 4	7.43	4.90, 2.50	
EcoRI 2	10.00		6.40, 3.50
BclI 6	2.42	2.17, 0.22[b]	
BclI 1	17.60		10.50, 6.40
ClaI 4	6.30		5.10, 1.25
BstEII 2	11.95	9.40, 2.61	
BstEII 3	6.30		4.90, 1.40
SstI 1	18.50	12.10, 6.10	
SstI 2	16.10		11.20, 4.70

a) Fragments cleaved by the single-site enzyme as detected in double digests.

b) Estimated fragment size.

Table 4. Cleavage products generated by the two-site enzymes PstI, SalI, BamHI, and SstII.

Fragment cleaved[a]	Size (kb)	Double digestion cleavage products PstI	SalI	BamHI	SstII
HincII 4[b]	6.18	5.65, 0.53			3.58, 2.60
HincII 3	8.04			7.80, 0.14[c]	
HincII 5	4.84			4.74, 0.10[c]	
BglII 1	11.90	6.50, 5.40		10.80, 0.80	
BglII 2	11.50	6.50, 4.90		9.80, 0.80	
HpaI 1	15.60	15.00, 0.60[c]	9.60, 6.20	9.51, 6.30	
HpaI 2	11.80	11.40, 0.60[c]	6.20, 5.50	6.30, 5.40	
EcoRI 1	13.50		11.80, 2.43	11.69, 2.54	
EcoRI 3	8.77	6.37, 2.14			
EcoRI 4	7.43	4.96, 2.14			
EcoRI 5	6.75		4.13, 2.43	3.99, 2.54	
ClaI 1	17.90	9.50, 8.10		15.80, 2.00	
ClaI 3	10.65	8.10, 2.55		8.55, 2.00	
BclI 4[b]	4.86			3.75, 1.07	4.66, 0.20[c]
BclI 5[b]	3.06	1.85, 1.20			
SstI 1	18.50	12.00, 3.10[b]			16.70, 0.90
SstI 4[b]	2.86			2.68, 0.18[c]	

a) Fragments cleaved by the two-site enzyme as detected in double digests.

b) Fragments represented in twice molar amount.

c) Estimated fragment size.

2.4 SstI, BstEII, AvaI, PvuII, and PstI restriction mapping

Both SstI and BstEII cut A. klebsiana mtDNA seven times. Single digests show only five bands on agarose gels because the bottom two bands in both digests are double bands (for e.g. see Fig. 22B, lanes c and d).

BstEII/SstI codigestion generated seven fragments that could be visualized on an agarose gel (Fig. 22A, lane d; Note: the top band was a partial digest product). The relative simplicity of the BstEII/SstI codigestion pattern allowed for the derivation of SstI and BstEII restriction maps by trial arrangement of fragments (Fig. 1a). The order of double digest fragments shown (Fig. 1a, middle track) agrees very well with the single digest fragments (Fig. 1a, top and bottom tracks).

Codigestion of the mtDNA by AvaI with SstI, and by AvaI with BstEII, confirmed that SstI1 and BstEII2 overlap since AvaI cleaved only these fragments, once each (Fig. 22A, lanes a and g; Fig. 22C, lane d). AvaI cleaved SstI1 into 12.1 and 6.1 kb fragments and BstEII2 into 9.4 and 2.61 kb fragments (Table 3). There are two possible orientations of SstI1 and BstEII2 relative to the AvaI site (Fig. 1bi, A+B and A+C). Only one orientation, A+C, which would generate a 12.01 kb fragment in the region of overlap,

is consistent with the BstEII/SstI codigest fragment sizes (Table 2b). Thus the orientation of SstI1 and BstEII2, as established by double digest analysis above, was confirmed, and the AvaI site was established.

Codigestion of the mtDNA by PvuII with SstI, and by PvuII with BstEII, confirmed that SstI2 and BstEII3 overlap since PvuII cleaved only these fragments, once each (Fig. 23A, lanes b and e). PvuII cleaved SstI2 into 11.2 and 4.7 kb fragments, and BstEII3 into 4.9 and 1.4 kb fragments (Table 3). There are two possible orientations of SstI2 and BstEII3 relative to the PvuII site (Fig. 1bii, X+Y and X+Z). In this case, both orientations would generate fragments consistent with the BstEII/SstI codigest fragment sizes (Table 2b). However, only one orientation, X+Z, supports the restriction map for SstI and BstEII already established (Fig. 1a). Although the PvuII digestions do not contribute anything to mapping at this point, the PvuII site itself is secure. This is repeatedly confirmed in the experiments that follow.

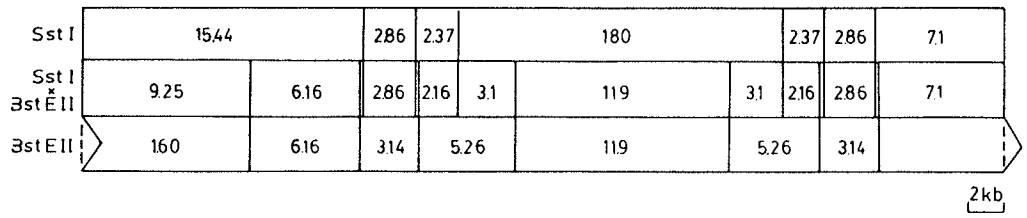
Codigestion of the mtDNA by PstI with SstI (Fig. 22A, lane f) showed that PstI cleaved SstI1 into a 12.0 kb fragment and a 3.1 kb double fragment (Table 4). This indicates that there are two PstI sites in the mtDNA, both within SstI1. Codigestion of the mtDNA by PstI with BstEII (Fig. 22A, lane b) showed that PstI did not cleave any BstEII fragments. There are two

possible orientations of the PstI sites within SstI1 (Fig. 1c, R and S) that would generate the cleavage products observed in the SstI/PstI codigestion (Table 4). Only one orientation, R, is consistent with the BstEII/PstI codigestion. In that case the two PstI sites would be close to the two BstEII sites defining the ends of BstEII2, such that in a BstEII/PstI codigestion PstI would not generate any detectable fragments. Thus the correct orientation of the PstI sites is R (Fig. 1c).

Fig 1d shows the completed SstI, BstEII, AvaI, PvuII, and PstI restriction maps, as established above. It is apparent from the map that reiterated sequences are present in the mtDNA (see Results section 2.3) and that they are inverted with respect to each other, separating the mt genome into two unequal-sized regions of unique sequence (Fig. 1d, arrows). The minimum size of the repeat regions (as mapped to this point is 8.4-8.5 kb.

Figure 1. Derivation of the SstI, BstEII, AvaI, PvuII, and PstI restriction maps. a) The SstI and BstEII fragment maps as deduced by analysis of the single and double digestion products. The molecular sizes (kb) of the fragments are shown. b) Relative orientation of the fragments SstI1 and BstEII2 to the AvaI site (i), and fragments SstI2 and BstEII3 to the PvuII site (ii), as deduced by analysis of double digest fragments generated by overlapping regions. c) Orientation of the PstI sites relative to fragments SstI1 and BstEII2 as deduced by analysis of SstI/PstI and BstEII/PstI codigestions. d) The completed SstI, BstEII, AvaI, PvuII, and PstI restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows.

a)

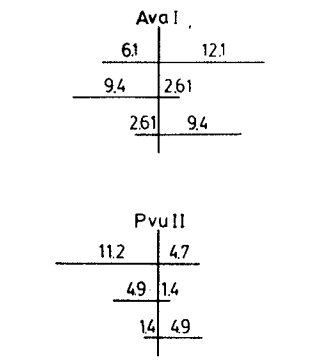


b)

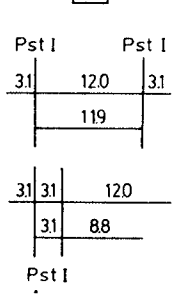
i)

ii)

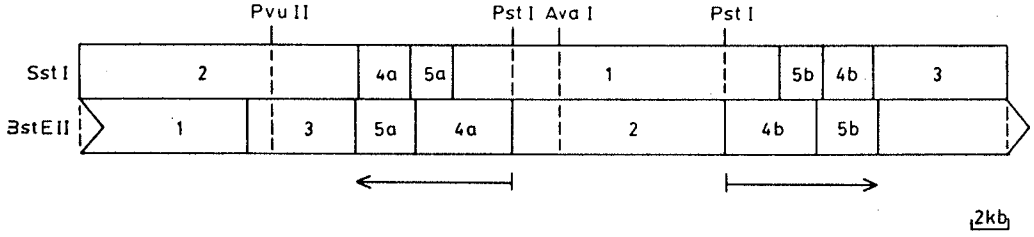
Fragment	Orient.	Double Digest Fragment Generated By Overlap: Fragment orient.	Predicted size (kb)	Observed size (kb)
Sst I 1	A			
BstE II 2	B	A + B	8.71	11.9
	C	A + C	12.01	
Sst I 2	X			
BstE II 3	Y	X + Y	6.3	6.16
	Z	X + Z	6.1	



c)



d)



2.5 ClaI restriction mapping

ClaI cut A. klebsiana mtDNA six times and generated six bands that were present in equimolar amounts (Fig. 23A, lane b). The absence of double bands in a single digest indicates that there can be no more than one ClaI site in each repeat region.

Codigestion of the mtDNA by ClaI with PstI (Fig. 23B, lane e) showed that PstI cleaved ClaI1 amd ClaI3 once each and generated fragments of 9.5 and 8.1 kb for ClaI1, and fragments of 8.1 and 2.55 kb for ClaI3 (Table 4). The generation of an 8.1 kb band in each case indicates that this portion of both of the ClaI fragments must be within the repeat regions, leaving the other two fragments (2.6 and 9.5 kb) to make up the small unique region between the two PstI sites (Fig. 2ai). There are two possible orientations of the ClaI site between the PstI sites (Fig. 2ai, A+B and A+C). The placement may be resolved by digestion of the ClaI fragments with AvaI. When this was done (data not shown), no new fragments were generated. This is in accord with orientation C in Fig 2ai since ClaI and AvaI sites are nearly coincident in this arrangement.

Codigestion of the mtDNA by ClaI with PvuII (Fig. 23B, lane d) showed that ClaI4 was cleaved by PvuII into 5.1 and 1.25 kb fragments (Table 4). ClaI4 must therefore overlap with SstI2 which contains the PvuII site. There are two possible orientations of

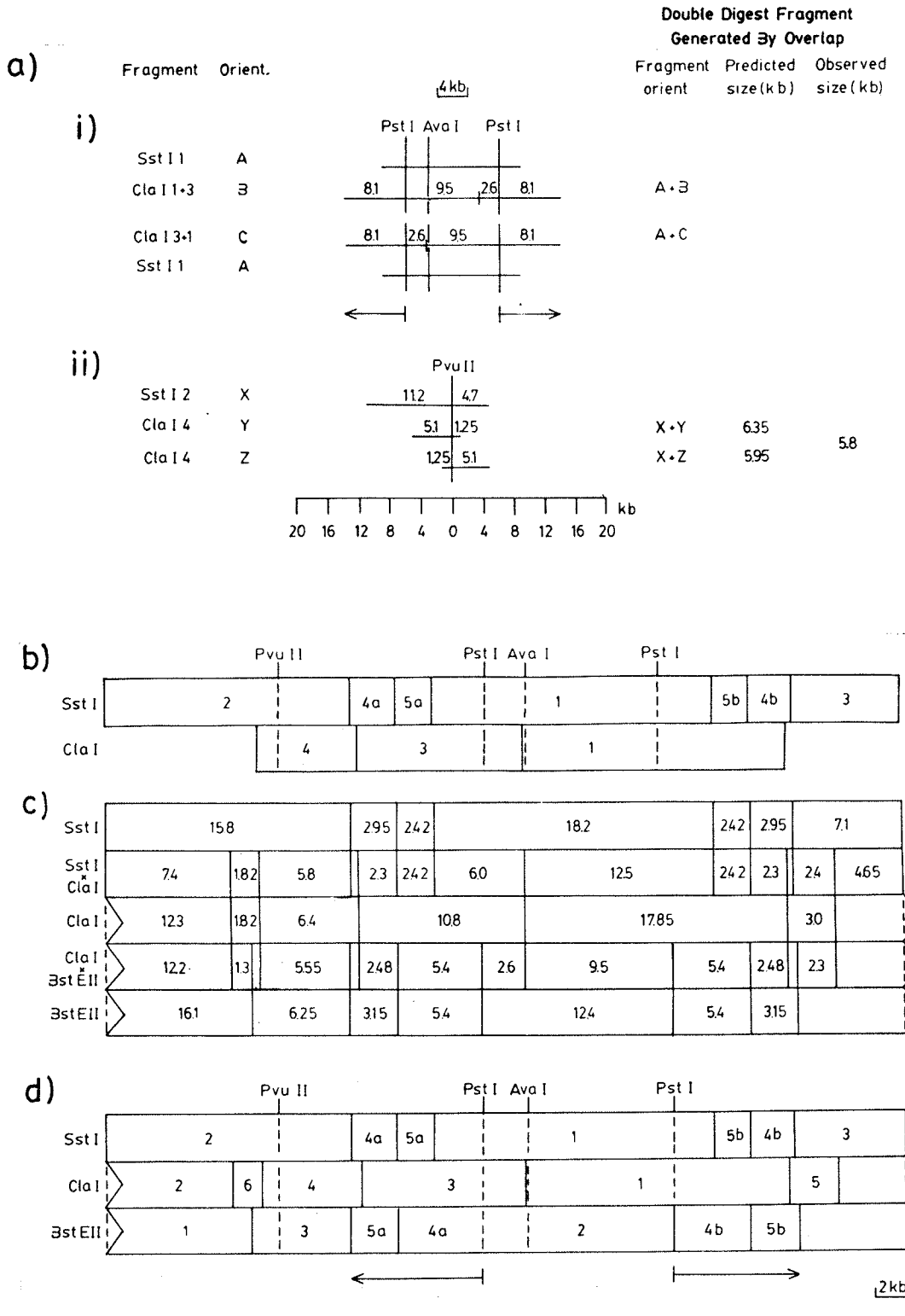
ClaI4 relative to the PvuII site and with respect to overlap with SstI2 (Fig. 2aii, X+Y and X+Z). Only one orientation, X+Z, which generates a 5.95 kb fragment in the region of overlap is consistent with the ClaI/SstI codigest fragment sizes (Table 2b). Thus X+Z was the correct orientation of ClaI4 (Fig. 2aii).

The fragment orders for ClaI1, ClaI3, and ClaI4, as established above, are shown in Fig 2b.

The three remaining ClaI fragments (ClaI2, 5, and 6) were then easily ordered by further analysis of the SstI/ClaI codigestion (Fig. 23C, lane h) as shown in Fig 2c. Analysis of the BstEII/ClaI codigestion (Fig. 27D, lane c), confirmed the ClaI fragment order, as shown in Fig 2c.

The completed ClaI fragment order, as established above, is shown in Fig 2d. Addition of ClaI sites to the map does not extend the minimum sizes of the inverted repeat regions (Fig. 2d, arrows) determined in the previous section.

Figure 2. Derivation of the ClaI restriction map. a) i) Orientation and order of fragments ClaI1 and ClaI3 relative to the PstI sites as deduced by analysis of ClaI/PstI and ClaI/AvaI codigestions. The repeat regions are shown by arrows. ii) Relative orientation of fragment ClaI4 to fragment SstI2 and the PvuII site as deduced by analysis of double digest fragments generated by overlapping regions. b) The map positions of fragments ClaI1, ClaI3, and ClaI4, as derived from data shown above. c) The completed ClaI fragment map as deduced by analysis of ClaI/SstI and ClaI/BstEII codigestion products. The molecular sizes (kb) of the fragments are shown. d) The completed ClaI restriction map as derived from data shown above. The minimum size of each inverted region is shown by arrows.


a)
Fragment
Orient.
Double Digest Fragment Generated By Overlap
Fragment orient
Predicted size (kb)
Observed size (kb)
4kb
i)
Pst I
Ava I
Pst I
Sst I 1
A
Cla I 1+3
B
8.1
9.5
2.6
8.1
A+B
Cla I 3+1
C
8.1
2.6
9.5
8.1
A+C
Sst I 1
A
ii)
Pvu II
Sst I 2
X
11.2
4.7
Cla I 4
Y
5.1
1.25
X+Y
6.35
5.8
Cla I 4
Z
1.25
5.1
X+Z
5.95
kb
20 16 12 8 4 0 4 8 12 16 20
b)
Pvu II
Pst I
Ava I
Pst I
Sst I
2
4a
5a
1
5b
4b
3
Cla I
4
3
1
c)
Sst I
15.8
2.95
2.42
18.2
2.42
2.95
7.1
Sst I
Cla I
7.4
1.82
5.8
2.3
2.42
6.0
12.5
2.42
2.3
2.4
4.65
Cla I
12.3
1.82
6.4
10.8
17.85
3.0
Cla I
BstEII
12.2
1.3
5.55
2.48
5.4
2.6
9.5
5.4
2.48
2.3
BstEII
16.1
6.25
3.15
5.4
12.4
5.4
3.15
d)
Pvu II
Pst I
Ava I
Pst I
Sst I
2
4a
5a
1
5b
4b
3
Cla I
2
6
4
3
1
5
BstEII
1
3
5a
4a
2
4b
5b
2kb

2.6 BglII restriction mapping

BglII cut A. klebsiana mtDNA eleven times and showed eight bands on agarose gels (Fig. 24B, lane c). BglII8 (0.85 kb) was not usually detectable as a double band as gauged by intensity of ethidium bromide fluorescence. However, the sum of the fragment sizes in a BglII single digest was more consistent with sums from other single digests when BglII8 was assigned a twice molar amount (Table 1). This assignment was eventually confirmed by mapping studies (below). BglII9 (0.2 kb) was not originally mapped because it was not detected in BglII digests run on agarose gels. BglII9 was mapped after being detected on polyacrylamide gels of BglII digests of a cloned fragment of mtDNA (see Results section 3.1). Its map position is included here for clarity of presentation.

Codigestion of the mtDNA by BglII with PvuII (Fig. 24A, lane g) showed that BglII4 was cleaved by PvuII into 4.58 and 2.35 kb fragments (Table 3). BglII4 must therefore overlap with ClaI4 since ClaI4 contains the PvuII site. There are two possible orientations of BglII4 relative to the PvuII site and with respect to the overlap with ClaI4 (Fig. 3ai, A+B and A+C). Only one orientation, A+C, which generates a 3.6 kb fragment in the region of overlap, is consistent with the BglII/ClaI codigest fragment sizes (Table 2a).

Thus, A+C is the correct orientation of BglII4 (Fig. 3ai).

Codigestion of the mtDNA by BglII with PstI (Fig. 24A, lane e) showed that PstI cleaved BglII1 and BglII2 once each and generated fragments of 6.5 and 5.4 kb for BglII1, and fragments of 6.5 and 4.7 kb for BglII2 (Table 4). The generation of a 6.5 kb band in each case indicates that this portion of both of the BglII fragments must be within the repeat regions, leaving the other two fragments (5.4 and 4.7 kb) within the small unique region (Fig. 3aii). Two orientations of BglII1 and BglII2 with respect to the PstI sites and relative to overlap with ClaI1 and ClaI3 are possible (Fig. 3aii, X+Y and X+Z). The correct orientation was deduced after analysis of a BglII/AvaI codigest. Codigestion of the mtDNA by BglII with AvaI (Fig. 24A, lane f) showed that AvaI cleaved BglII into 9.7 and 1.88 kb fragments (Table 3). This is only possible when the orientation of BglII1 and BglII2 is X+Z (Fig. 3aii), because only in this orientation does BglII2 span the AvaI site to yield 9.7 and 1.88 kb fragments upon codigestion (Table 3).

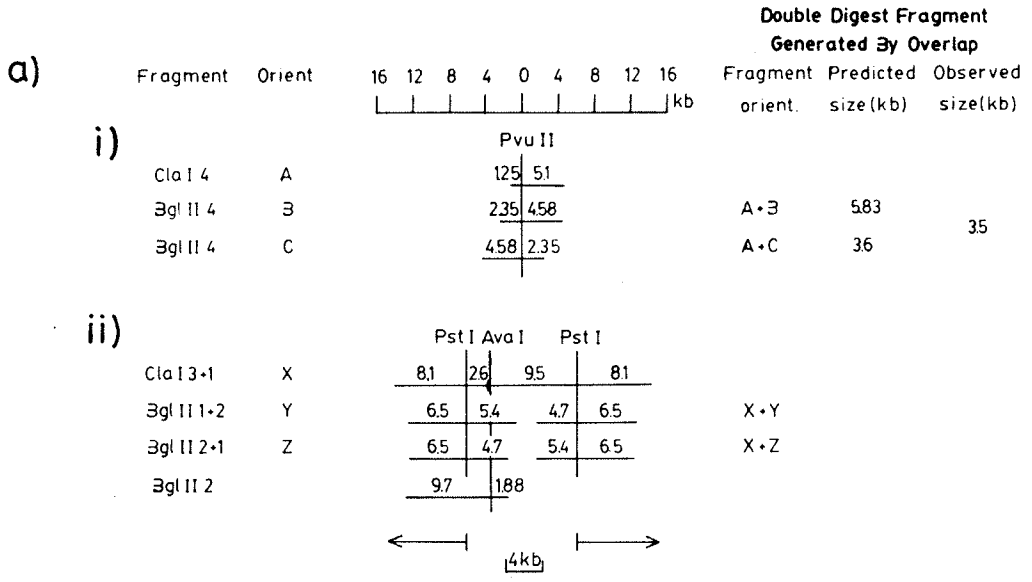
The fragment orders for BglII1, BglII2, and BglII4, as established above, are shown in Fig 3b.

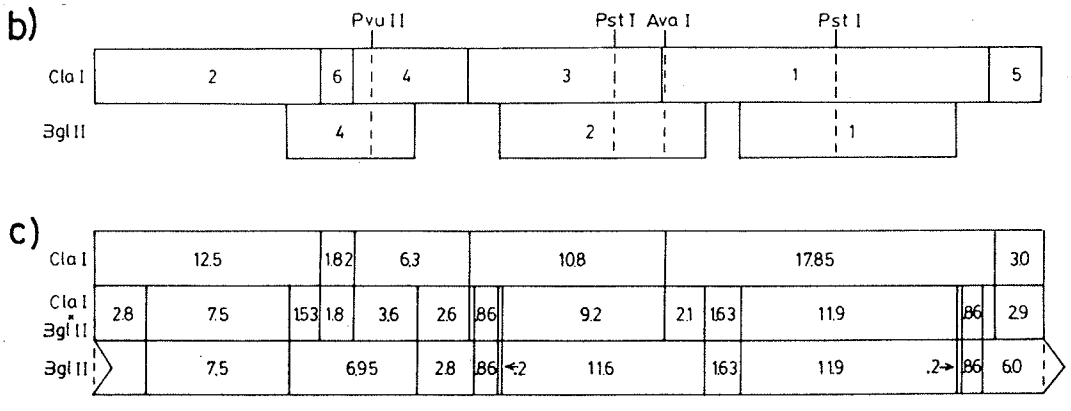
The rest of the BglII fragments (BglII3, 5, 6, 7, 8, and 9) were then easily ordered by further analysis of the BglII/ClaI codigest (Fig. 24B, lane a) as shown in Fig 3c.

BglII9 (0.2 kb) was detected only after polyacrylamide gel analysis of BglII digests of a cloned mtDNA fragment (BclI4) that had been mapped to the repeat regions (see Results sections 2.11 and 3.1). The map positions of BglII9a and BglII9b are shown in Fig 3c.

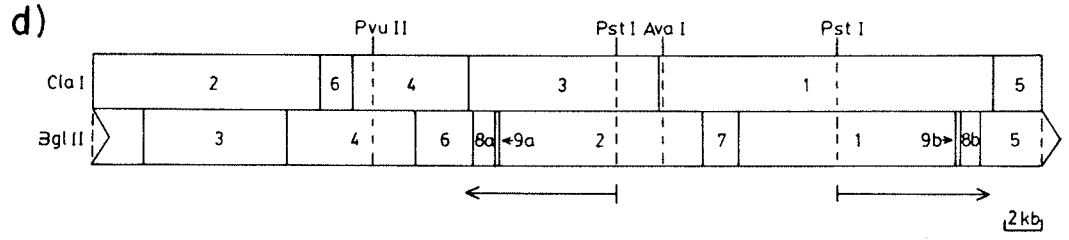
The completed BglII fragment order, as established above, is shown in Fig 3d. Addition of BglII sites to the map does not extend the minimum sizes of the inverted repeat regions (Fig. 3d, arrows) determined previously (Results section 2.4).

Figure 3. Derivation of the BglII restriction map. a) i) Relative orientation of fragment BglII4 to the PvuII site as deduced by analysis of double digest fragments generated by overlapping regions. ii) Orientation and order of fragments BglII1 and BglII2 relative to the PstI sites as deduced by analysis of BglII/PstI and BglII/AvaI codigestions. The repeat regions are shown by arrows. b) The map positions of fragments BglII1, BglII2, and BglII4, as derived from data shown above. c) The completed BglII fragment map as deduced by analysis of BglII/ClaI codigestion products. The molecular sizes (kb) of the fragments are shown. d) The completed BglII restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows.


a)
Fragment
Orient
16 12 8 4 0 4 8 12 16
kb
Double Digest Fragment Generated By Overlap
Fragment orient.
Predicted size(kb)
Observed size(kb)
i)
Pvu II
Cla I 4
A
12.5 5.1
Bgl II 4
B
2.35 4.58
A+B
5.83
3.5
Bgl II 4
C
4.58 2.35
A+C
3.6
ii)
Pst I Ava I Pst I
Cla I 3+1
X
8.1 2.6 9.5 8.1
Bgl II 1+2
Y
6.5 5.4 4.7 6.5
X+Y
Bgl II 2+1
Z
6.5 4.7 5.4 6.5
X+Z
Bgl II 2
9.7 1.88
4kb


b)
Pvu II
Pst I Ava I
Pst I
Cla I
2 6 4 3 1 5
Bgl II
4 2 1

c)
Cla I
12.5 1.8 2 6.3 10.8 17.8 5 30
Cla I
Bgl II
2.8 7.5 1.5 3 1.8 3.6 2.6 .8 6 9.2 2.1 1.6 3 11.9 .8 6 2.9
Bgl II
7.5 6.9 5 2.8 .8 6 .2 11.6 1.6 3 11.9 .2 .8 6 6.0


d)
Pvu II
Pst I Ava I
Pst I
Cla I
2 6 4 3 1 5
Bgl II
3 4 6 8a 9a 2 7 1 9b 8b 5
2kb

2.7 HincII restriction mapping

Digestion of A. klebsiana mtDNA with HincII showed nine bands on agarose gels (see Fig. 25B, lane d); HincII4 was a double band and HincII10 has not been visualized but was postulated to occur, as discussed below.

Codigestion of the mtDNA by HincII with PvuII (Fig. 25B, lane f) showed that HincII3 was cleaved by PvuII into 7.55 and 0.41 kb fragments (Table 3). HincII3 must therefore overlap with ClaI4 since this fragment contains the PvuII site. There are two possible orientations of HincII3 relative to the PvuII site and with respect to overlap with ClaI4 (Fig. 4ai, A+B and A+C). Only one orientation, A+B, which generates a 5.42 kb fragment in the region of overlap, is consistent with the HincII/ClaI codigest fragment sizes (Table 2b). Thus, A+B is the correct orientation of HincII3 (Fig. 4ai).

Codigestion of the mtDNA by HincII with PstI (Fig. 25B, lane e) showed that PstI cleaved HincII4, a double band, into 5.65 and 0.53 kb fragments, also double bands (Table 4). Since at least one end of HincII4 must be in the repeat region, only the two symmetrical arrangements shown in Fig 4aii (X or Y) are possible. Since AvaI does not cleave HincII4 (Fig. 25C, lane b), arrangement Y is eliminated.

The fragment orders for HincII3, HincII4a, and HincII4b, as established above, are shown in Fig 4b.

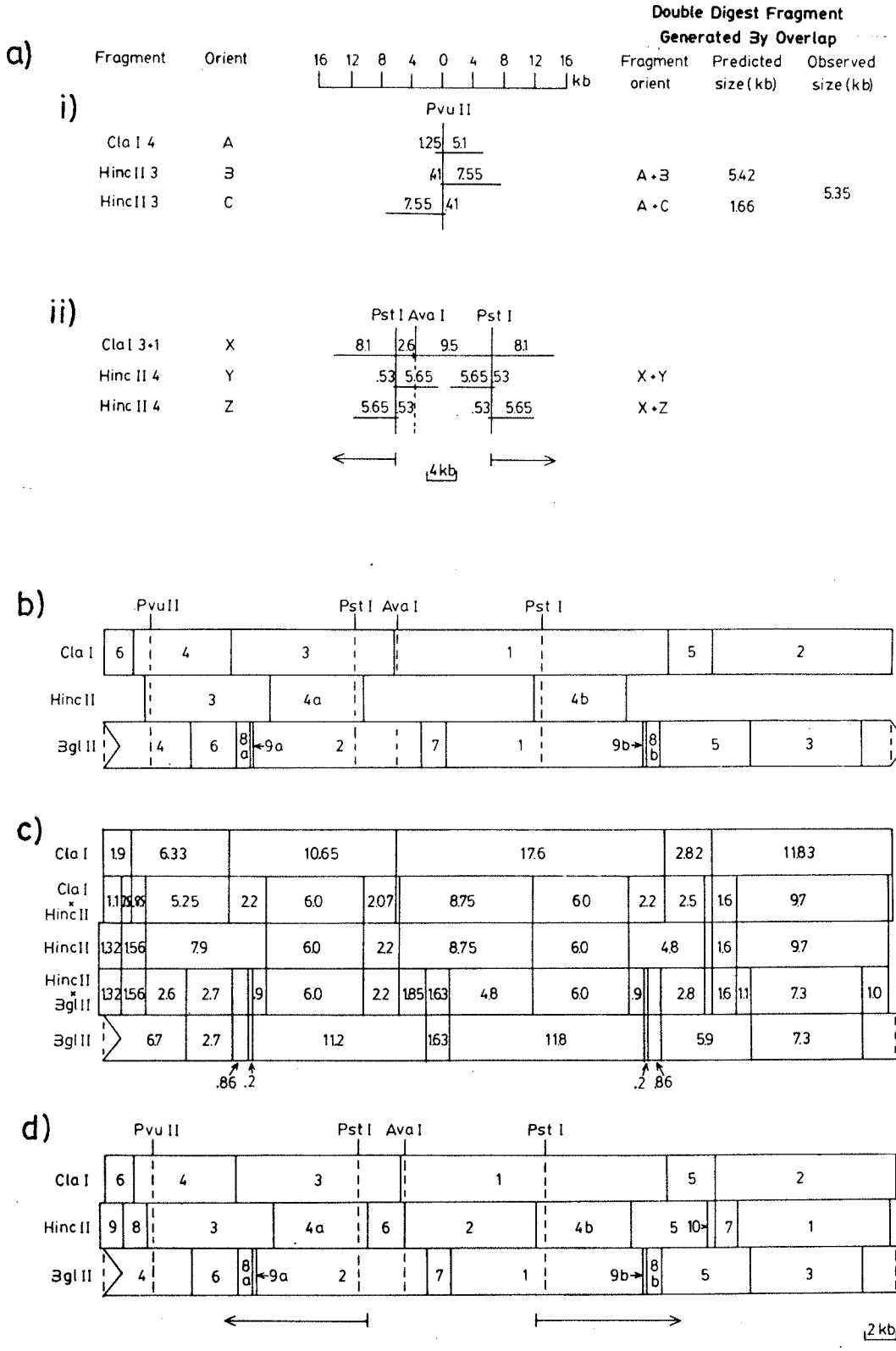
The rest of the HincII fragments (HincII1, 2, 5, 6, 7, 8, 9, and 10) were ordered by further analysis of the HincII/ClaI codigest (Fig. 24B, lane g; Fig. 25A, lane b), and by analysis of the HincII/BglII codigest (Fig. 24A, lane c; Fig. 24B, lane d), as shown in Fig 4c. Also helpful was the HincII/AvaI codigest (Fig. 25C, lane b) which showed that AvaI did not cleave any HincII fragment to yield a fragment large enough for detection in agarose. It was concluded that a HincII site was close to the AvaI site. HincII1 was not cleaved in the HincII/ClaI codigest (see Fig. 25A) and therefore must have been contained within ClaI2, the only remaining ClaI fragment large enough to contain it. HincII2 was also not cleaved in the HincII/ClaI codigest (see Fig. 25A) and therefore it must be contained within ClaI1 adjacent to HincII4b. HincII6 (2.2 kb) fits the space between HincII4a and HincII2 (Fig. 4c). Since HincII8 (1.56 kb) and HincII9 (1.32 kb), as well as ClaI6 (1.9 kb), were all cleaved in the HincII/ClaI codigest (Fig. 25A), analysis of the codigest fragment sizes (Table 2b) allowed placing of HincII8 next to HincII3, and HincII9 next to HincII8 (Fig. 4c). HincII5 (4.8 kb) was mapped next to HincII4b (Fig. 4c) by analysis of the HincII/ClaI and HincII/BglII codigest fragment sizes (Tables 2a and 2b).

HincII7 (1.6 kb) was not cleaved in the HincII/ClaI codigest (Fig. 25A), and thus must have been conserved within ClaI2 next to HincII1. The order of HincII1 and HincII7 within ClaI2 was established by reference to the HincII/BglII codigest fragment sizes (Table 2a, Fig. 4c). The absence of a ClaI site in HincII7 led to the postulate that a tenth HincII fragment (approximately 0.3 kb) occurs between HincII5 and HincII7 (Fig. 4c). The existence of HincII10 is supported by the observation that the sum of the HincII/BglII codigestion fragments corresponding to BglII5 (Fig. 4c) has a significant deficit.

The completed HincII fragment order, as established above, is shown in Fig 4d.

At this point the repeat regions could be extended into the small unique region up to the ends of HincII4a and HincII4b (Fig. 4d, arrows). The minimum size of each repeat region could then be estimated at approximately 9 kb.

Figure 4. Derivation of the HincII restriction map. a) i) Relative orientation of fragment HincII3 to fragment ClaI4 and the PvuII site as deduced by analysis of double digest fragments generated by overlapping regions. ii) Orientation of fragments HincII4a and HincII4b relative to the PstI sites as deduced by analysis of HincII/PstI and HincII/AvaI codigestions. The repeat regions are shown by arrows. b) The map positions of fragments HincII3, HincII4a, and HincII4b, as derived from data shown above. c) The completed HincII fragment map as deduced by analysis of HincII/ClaI and HincII/BglII codigestion products. The molecular sizes (kb) of the fragments are shown. d) The completed HincII restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows.


a)
Fragment
Orient
16 12 8 4 0 4 8 12 16 kb
Double Digest Fragment Generated By Overlap
Fragment orient
Predicted size (kb)
Observed size (kb)
i)
Pvu II
Cla I 4
A
1.25 5.1
Hinc II 3
B
4.1 7.55
A + B
5.42
5.35
Hinc II 3
C
7.55 4.1
A + C
1.66
ii)
Pst I Ava I Pst I
Cla I 3+1
X
8.1 2.6 9.5 8.1
Hinc II 4
Y
.53 5.65 5.65 .53
X + Y
Hinc II 4
Z
5.65 .53 .53 5.65
X + Z
4kb
b)
Pvu II
Pst I Ava I
Pst I
Cla I
Hinc II
Bgl II
c)
Cla I
Cla I × Hinc II
Hinc II
Hinc II × Bgl II
Bgl II
d)
Pvu II
Pst I Ava I
Pst I
Cla I
Hinc II
Bgl II
2 kb

2.8 HpaI and SalI restriction mapping

HpaI cut A. klebsiana mtDNA seven times and generated seven bands that were present in equimolar amounts (Fig. 26A, lane f). The absence of double bands in a single digest indicated that there could be no more than one HpaI site in each of the repeat regions.

Since HpaI recognizes the sequence 5'-GTTAAC-3' (Sharp et al, 1973; Garfin and Goodman, 1974), and HincII recognizes the sequence 5'-GT(C/T)(A/G)AC-3' (Smith and Wilcox, 1970; Kelly and Smith, 1970), HincII will recognize all HpaI sites (but not vice versa) so that HincII and HpaI digests of the mtDNA would be expected to share some common fragments. Comparison of HpaI and HincII digests of the mtDNA showed the following pairs of common fragments: HpaI3 and HincII1, HpaI4 and HincII2, HpaI5 and HincII6, HpaI6 and HincII7, and HpaI7 and HincII9 (compare lanes c and d, Fig. 25C). Thus, the map positions of the HpaI fragments were deduced from the HincII fragment map, as shown in Fig 5ai. HpaI1 and HpaI2 then fit naturally into the two remaining spaces left in the map (Fig. 5aii, iii). In a HpaI/PstI codigestion (Fig. 25C, lane e), PstI cleaved 0.6 kb fragments from each end of HpaI1 and HpaI2 (Table 4) as was predicted from the completed HpaI map (see Fig. 5aiii). In a HpaI/PvuII codigestion

(Fig. 25C, lane f), PvuII cleaved HpaI1 into 13.5 and 2.05 kb fragments (Table 3) as was predicted from the completed HpaI map (see Fig. 5aiii), thus also confirming the HpaI1 map position as established above.

Codigestion of the mtDNA by HpaI with SalI (Fig. 26B) showed that SalI cleaved HpaI1 and HpaI2 once each and generated fragments of 9.6 and 6.2 kb for HpaI1, and 6.2 and 5.5 kb for HpaI2 (Table 4; Fig. 5bi). The 6.2 kb double band indicated that each repeat contained one SalI site. Since SalI recognizes the sequence 5'-GTCGAC-3' (Arrand et al, 1978), HincII will recognize all SalI sites (but not vice versa; see above for the HincII recognition sequence). Therefore, since there is only one HincII site in each repeat that is not also a HpaI site (see Fig. 5aiii), these two HincII sites must be the SalI sites (see Fig. 5bii). Thus, the 6.2 kb double band generated in the HpaI/SalI codigestion (Table 4; Fig. 26B) was identical to HincII4 (see Fig. 5bi).

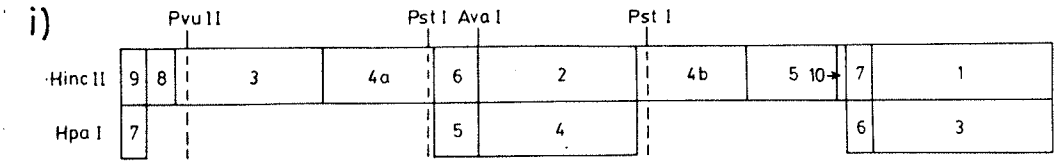
Thus there are seven HincII/HpaI sites (5'-GTTAAC-3'), two HincII/SalI sites (5'-GTCGAC-3'), and two HincII only sites (5'-GTTGAC-3' or 5'-GTCAAC-3') in A. klebsiana mtDNA.

These sites, as established above, are shown in Fig 5bii.

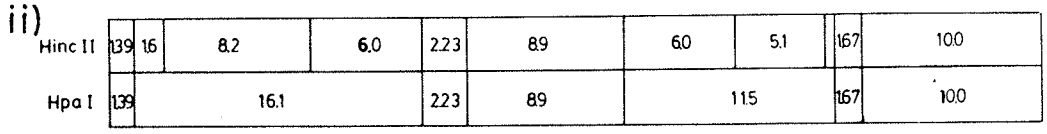
Figure 5. Derivation of the HpaI and SalI restriction maps. a) Derivation of the HpaI restriction map. i) The map positions of fragments HpaI3, HpaI4, HpaI5, HpaI6, and HpaI7, as deduced from those fragments from HpaI and HincII single digests that had identical electrophoretic mobilities. ii) The completed HpaI fragment map as deduced by analysis of HpaI and HincII fragment sizes (kb; shown). iii) The completed HpaI restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows. b) Derivation of the SalI restriction map. i) Cleavage of fragments HpaI1 and HpaI2 by SalI. ii) The map positions of the SalI sites as deduced from the HpaI/SalI codigestion and the HpaI and HincII restriction maps. The minimum size of each inverted repeat region is shown by arrows.

a)

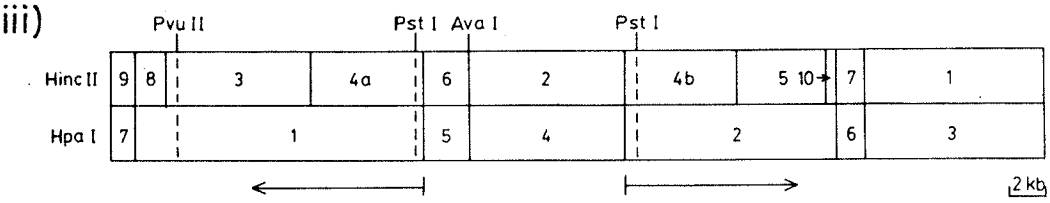
i)



ii)

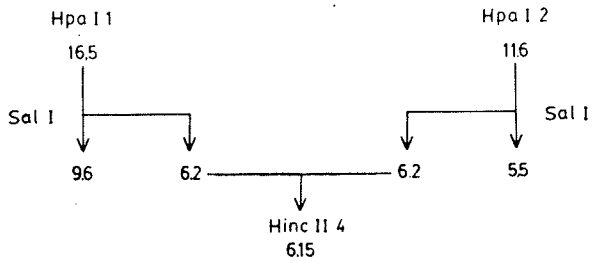


iii)

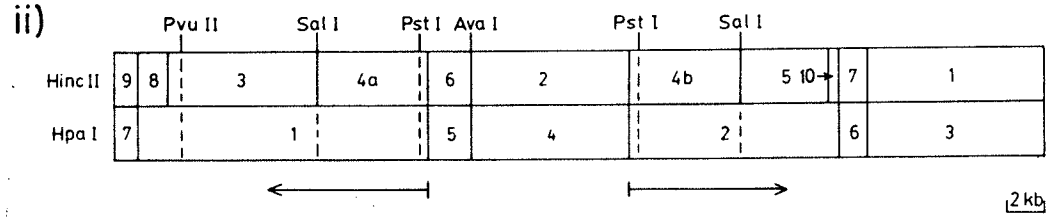


b)

i)



ii)



2.9 EcoRI restriction mapping

EcoRI cut A. klebsiana mtDNA nine times and generated eight bands as visualized on agarose gels (Fig. 26A, lane d). The bottom band, EcoRI8, is a double band.

Codigestion of the mtDNA by EcoRI with PvuII (digest not shown) showed that PvuII cleaved EcoRI2 into 6.4 and 3.5 kb fragments (Table 3). EcoRI2 must therefore overlap with HincII3 since HincII3 contains the PvuII site. There are two possible orientations of EcoRI2 relative to the PvuII site and with respect to overlap with HincII3 (Fig. 6ai, A+B and A+C). Only one orientation, A+B, which generates a 3.91 kb fragment in the region of overlap, is consistent with the EcoRI/HincII codigestion fragment sizes (Table 2b). Thus, A+B is the correct orientation of EcoRI2 (Fig. 6ai).

Codigestion of the mtDNA by EcoRI with PstI (digest not shown) showed that PstI cleaved EcoRI3 and EcoRI4 once each and generated fragments of 6.37 and 2.14 kb for EcoRI3, and fragments of 4.9 and 2.14 kb for EcoRI4 (Table 4). The generation of the 2.14 kb double band indicated that there was an EcoRI site in each repeat region, with the two possible arrangements seen in Fig 6aii. Codigestion of the mtDNA by EcoRI with AvaI (digest not shown) showed that AvaI cleaved EcoRI4 into 4.9 and 2.0 kb fragments (Table 3; Fig. 6aii).

The correct orientation of EcoRI3 and EcoRI4 must be X+Z (Fig. 6aii), as only in this orientation does EcoRI4 span the AvaI site consistent with the EcoRI/AvaI codigest fragment sizes (Table 3).

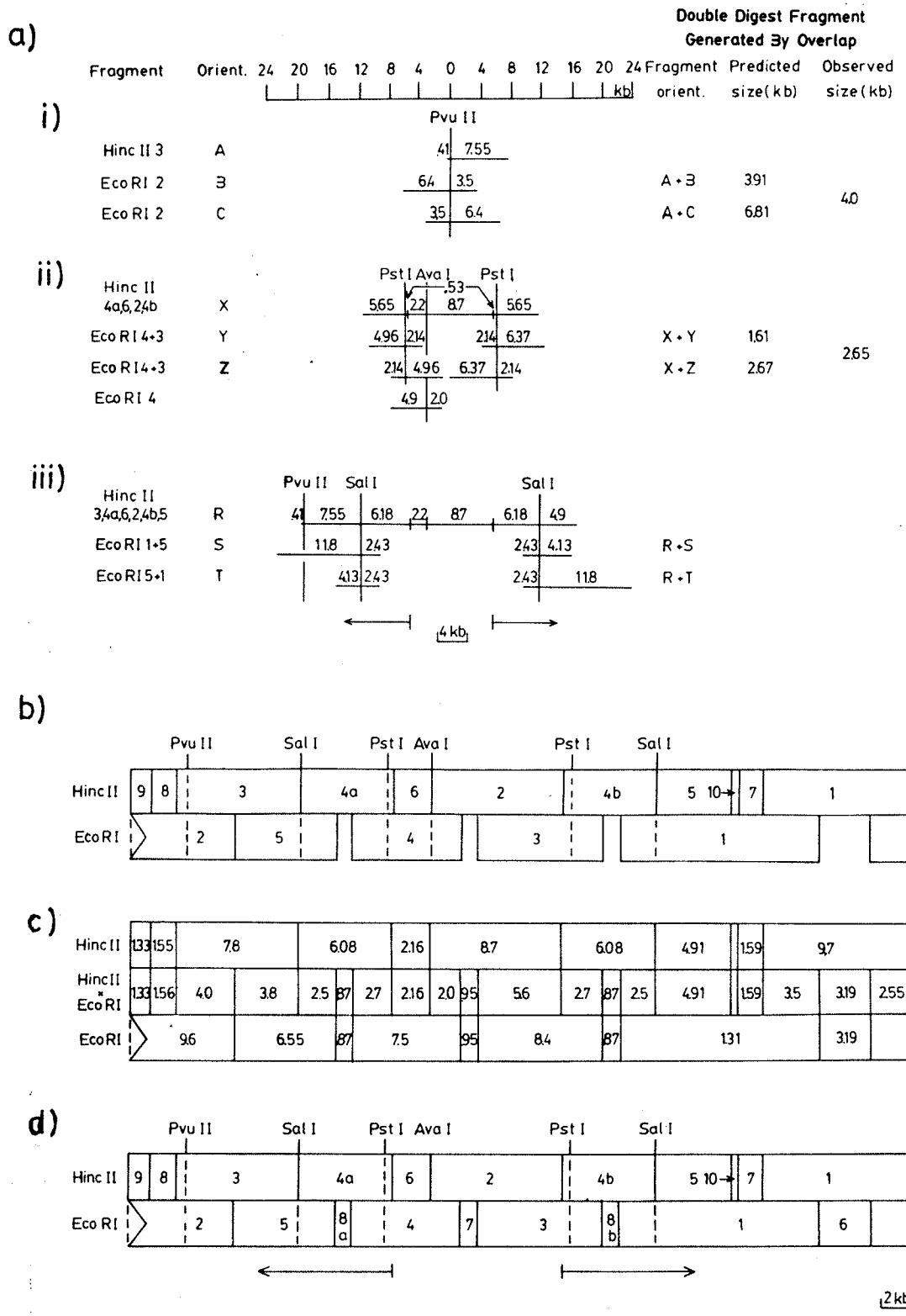
Codigestion of the mtDNA by EcoRI with SalI (Fig. 26A, lane b) showed that SalI cleaved EcoRI1 and EcoRI5 once each and generated fragments of 11.80 and 2.43 kb for EcoRI1, and fragments of 4.13 and 2.43 kb for EcoRI5 (Table 4). The generation of the 2.43 kb double band indicated that there was another EcoRI site in each repeat region. There are two possible orientations of EcoRI1 and EcoRI5 with respect to the SalI sites (Fig. 6aiii, X+Y and X+Z). Since it is EcoRI2 that is cleaved by PvuII (Fig. 6ai), R+S, in which EcoRI1 spans the PvuII site (Fig. 6aiii), is eliminated. Thus, R+T is the correct orientation of EcoRI1 and EcoRI5 (Fig. 6aiii).

The fragment orders for EcoRI1, EcoRI2, EcoRI3, EcoRI4, and EcoRI5, as established above, are shown in Fig 6b.

The remaining EcoRI fragments (EcoRI6, 7 and 8) were then easily ordered by further analysis of the EcoRI/HincII codigestion (Fig. 25B, lane c) as shown in Fig 6c.

The completed EcoRI fragment order, as established above, is shown in Fig 6d.

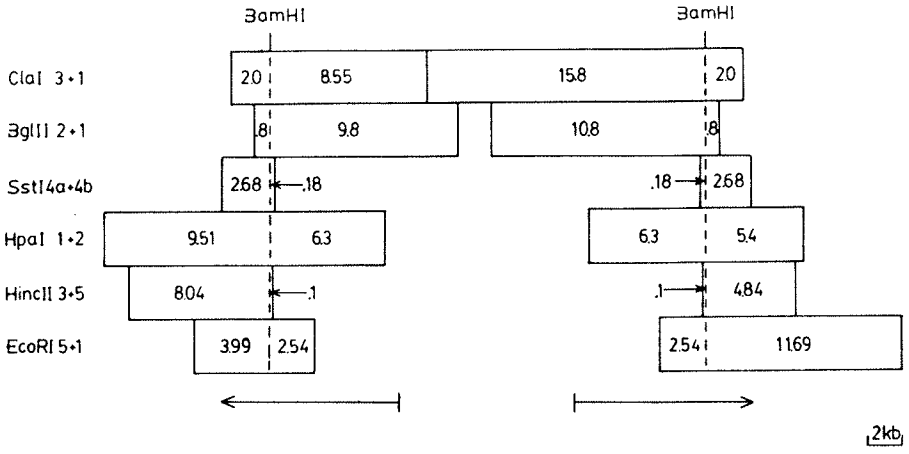
Figure 6. Derivation of the EcoRI restriction map. a) i) Relative orientation of fragment EcoRI2 to fragment HincII3 and the PvuII site as deduced by analysis of the double digest fragments generated by overlapping regions. ii) Orientation and order of fragments EcoRI3 and EcoRI4 relative to the PstI sites as deduced by analysis of EcoRI/PstI and EcoRI/AvaI codigestions, and by analysis of EcoRI/HincII codigest fragments generated by overlapping regions. iii) Orientation and order of fragments EcoRI1 and EcoRI5 relative to the SalI sites as deduced by analysis of EcoRI/SalI and EcoRI/PvuII codigestions. The repeat regions are shown by arrows. b) The map positions of fragments EcoRI1, EcoRI2, EcoRI3, EcoRI4, and EcoRI5, as derived from data shown above. c) The completed EcoRI fragment map as deduced by analysis of EcoRI/HincII codigestion products. The molecular sizes (kb) of the fragments are shown. d) The completed EcoRI restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows.


a)
Double Digest Fragment Generated By Overlap
Fragment
Orient.
24 20 16 12 8 4 0 4 8 12 16 20 24 kb
Fragment orient.
Predicted size(kb)
Observed size(kb)
i)
Pvu II
Hinc II 3
A
4.1
7.55
Eco RI 2
B
6.4
3.5
A+B
3.91
4.0
Eco RI 2
C
3.5
6.4
A+C
6.81
ii)
Hinc II 4a,6,2,4b
X
Pst I
Ava I
.53
Pst I
5.65
2.2
8.7
5.65
Eco RI 4+3
Y
4.96
2.14
2.14
6.37
X+Y
1.61
2.65
Eco RI 4+3
Z
2.14
4.96
6.37
2.14
X+Z
2.67
Eco RI 4
4.9
2.0
iii)
Hinc II 3,4a,6,2,4b,5
R
Pvu II
Sal I
Sal I
4.1
7.55
6.18
2.2
8.7
6.18
4.9
Eco RI 1+5
S
1.18
2.43
2.43
4.13
R+S
Eco RI 5+1
T
4.13
2.43
2.43
1.18
R+T
4 kb
b)
Pvu II
Sal I
Pst I
Ava I
Pst I
Sal I
Hinc II
9
8
3
4a
6
2
4b
5
10
7
1
Eco RI
2
5
4
3
1
c)
Hinc II
1.33
1.55
7.8
6.08
2.16
8.7
6.08
4.91
1.59
9.7
Hinc II + Eco RI
1.33
1.56
4.0
3.8
2.5
.87
2.7
2.16
2.0
.95
5.6
2.7
.87
2.5
4.91
1.59
3.5
3.19
2.55
Eco RI
9.6
6.55
.87
7.5
.95
8.4
.87
13.1
3.19
d)
Pvu II
Sal I
Pst I
Ava I
Pst I
Sal I
Hinc II
9
8
3
4a
6
2
4b
5
10
7
1
Eco RI
2
5
8a
4
7
3
8b
1
6
2 kb

2.10 BamHI restriction mapping

Codigestions of A. klebsiana mtDNA by various multisite enzymes with BamHI showed that BamHI recognized the following fragments by cleaving them once each (see Table 4); SstI4a and SstI4b (Fig. 22B, lane b), ClaI1 and ClaI3 (Fig. 23A, lane f), HincII3 and HincII5 (Fig. 24A, lane a), BglII1 and BglII2 (Fig. 24B, lane b), EcoRI1 and EcoRI5 (Fig. 26A, lane c), and HpaI1 and HpaI2 (Fig. 26A, lane g). Thus, the BamHI codigestions indicated that there were two BamHI sites in the mtDNA, with one site located in one repeat in the common sequence shared by ClaI3, BglII2, SstI4a, HpaI1, HincII3, and EcoRI5, and with the other site in the other repeat in the common sequence shared by ClaI1, BglII1, SstI4b, HpaI2, HincII5, and EcoRI1. Based on the BamHI codigest fragment sizes (Table 4) and on the established map positions of the recognized fragments, the two BamHI sites were mapped as shown in Fig 7.

Figure 7. Derivation of the BamHI restriction map. Shown are the BamHI sites on a partial restriction map consisting of the (previously mapped) fragments cleaved in codigestions by BamHI with ClaI, BglII, SstI, HpaI, HincII, and EcoRI. The sites were mapped by analysis of the codigest fragment sizes. The minimum size of each inverted repeat region is shown by arrows.


BamHI
BamHI
ClaI 3•1
BglII 2•1
SstI4a•4b
HpaI 1•2
HincII 3•5
EcoRI 5•1
2.0
8.55
15.8
2.0
.8
9.8
10.8
.8
2.68
.18
.18
2.68
9.51
6.3
6.3
5.4
8.04
.1
.1
4.84
3.99
2.54
2.54
11.69
2kb

2.11 BclI restriction mapping

BclI cut A. klebsiana mtDNA nine times and generated seven bands as visualized on agarose gels (Fig. 26C, lane c). BclI4 and BclI5 are double bands.

Codigestion of the mtDNA by BclI with PvuII (Fig. 26D, lane c) showed that PvuII cleaved BclI1 into 10.5 and 6.4 kb fragments (Table 3). BclI1 must therefore overlap HincII3 since HincII3 contains the PvuII site. There are two possible orientations of BclI1 relative to the PvuII site and with respect to overlap with HincII3 (Fig. 8ai, A+B and A+B). Only one orientation, A+B, which generates a 6.81 kb fragment in the region of overlap, is consistent with the BclI/HincII codigest fragment sizes (Table 2a). Thus, A+B is the correct orientation of BclI1 (Fig. 8ai).

Codigestion of the mtDNA by BclI with PstI (Fig. 26D, lane e) showed that PstI cleaved BclI5, a double band, into 1.85 and 1.20 kb fragments (Table 4). BclI5a and BclI5b must therefore overlap part of HincII4a and HincII4b, since these fragments contain the PstI sites. There are two possible orientations of BclI5a and BclI5b relative to the PstI sites and with respect to overlap with HincII4a and HincII4b (Fig. 8aii, X+Y and X+Z). Only one orientation, X+Y, which generates a 2.38 kb double band from the regions of overlap, is consistent with the BclI/HincII codigest fragment sizes (Table 2a). Thus, X+Y is the correct orientation for

BclI5a and BclI5b (Fig. 8aii).

Codigestion of the mtDNA by BclI with AvaI (Fig. 26D, lane d) showed that AvaI cleaved BclI6 into 2.17 and 0.22 kb fragments (Table 3). There are two possible orientations of BclI6 relative to the AvaI site (Fig. 8aii, X+M and X+N). Orientation X+N cannot be correct because in this orientation BclI6 would overlap BclI5b (see figure 8aii). Thus, X+N must be the correct orientation of BclI6 (Fig. 8aii).

Codigestion of the mtDNA by BclI with BamHI (Fig. 26D, lane f) showed that BamHI cleaved BclI4, a double band, into 3.75 and 1.07 kb fragments (Table 4). BclI4a and BclI4b must therefore overlap part of HincII4a and HincII4b, since these fragments contain the BamHI sites. There are two possible orientations of BclI4a and BclI4b relative to the BamHI sites and with respect to overlap with HincII4a and HincII4b (Fig. 8aiii, R+S and R+T). Only one orientation, R+T, which generates a 3.65 kb double band from the regions of overlap, is consistent with the BclI/HincII codigest fragment sizes (Table 2a). Thus, R+T is the correct orientation of BclI4a and BclI4b (Fig. 8aiii).

The fragment orders of BclI1, BclI4, BclI5, and BclI6, as established above, are shown in Fig 8b.

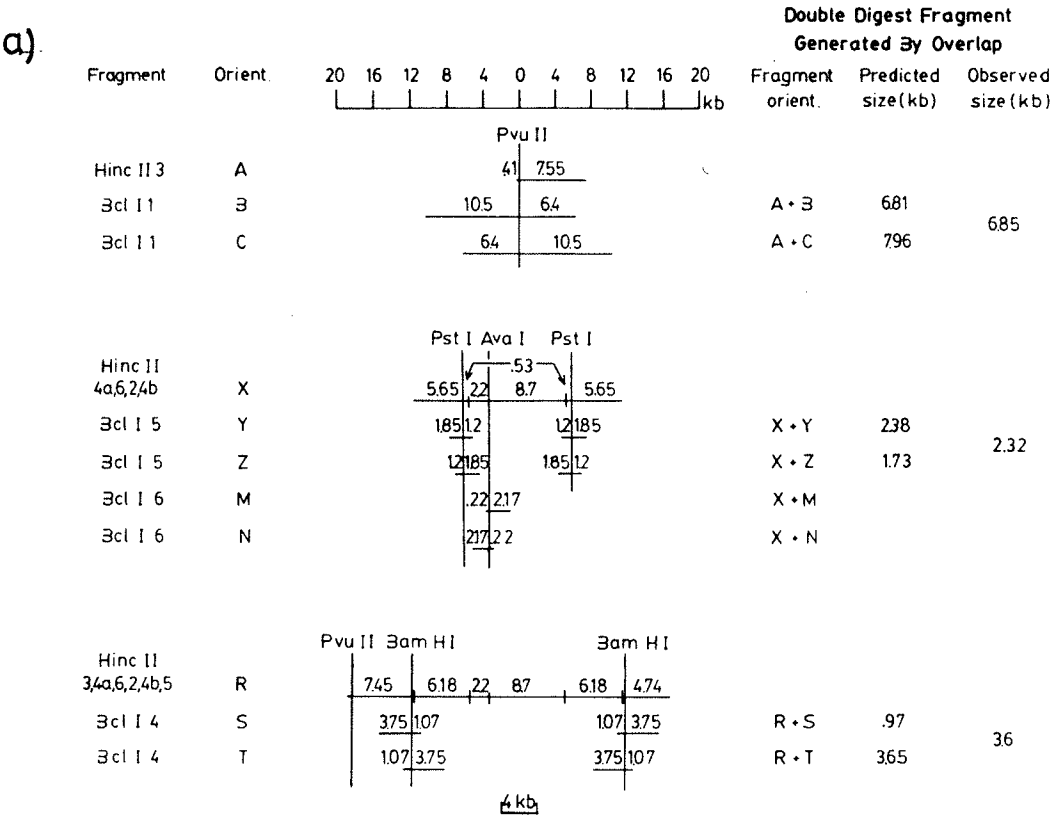
The three remaining BclI fragments (BclI2, 3, and 7) were ordered by further analysis of the BclI/HincII codigestion (Fig. 26C, lane b), as shown in Fig 8c. BclI2 (8.8 kb) must lie in the appropriately-sized space between BclI4b and BclI1; BclI3 (5.5 kb) in the appropriately-sized space between BclI6 and BclI5b; and BclI7 (1.3 kb) in the appropriately-sized space between BclI5a and BclI6.

The complete BclI fragment order, as established above, is shown in Fig 8d.

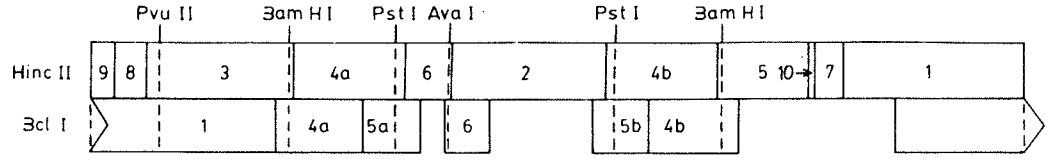
At this point the repeat regions could be extended into the small unique region up to the ends of BclI5a and BclI5b (Fig. 8d, arrows). The minimum size of each repeat region could then be estimated at approximately 9.7 kb.

Figure 8. Derivation of the BclI restriction map.
a) i) Relative orientation of fragment BclI1 to fragment HincII3 and the PvuII site as deduced by analysis of the double digest fragments generated by overlapping regions. ii) Orientation and order of fragments BclI5a and BclI5b relative to the PstI sites as deduced by analysis of the BclI/PstI codigestion, and by analysis of the BclI/HincII codigest fragments generated by overlapping regions; and relative orientation of fragment BclI6 to the AvaI site as deduced by analysis of the BclI/AvaI codigestion and process of elimination. iii) Relative orientation of fragments BclI4a and BclI4b to the BamHI sites as deduced by analysis of BclI/HincII codigest fragments generated by overlapping regions. b) The map positions of fragments BclI1, BclI4a, BclI4b, BclI5a, BclI5b, and BclI6, as derived from data shown above.
c) The completed BclI fragment map as deduced by analysis of BclI/HincII codigestion products.
The molecular sizes (kb) of the fragments are shown.
d) The completed BclI restriction map as derived from data shown above. The minimum size of each inverted repeat region is shown by arrows.

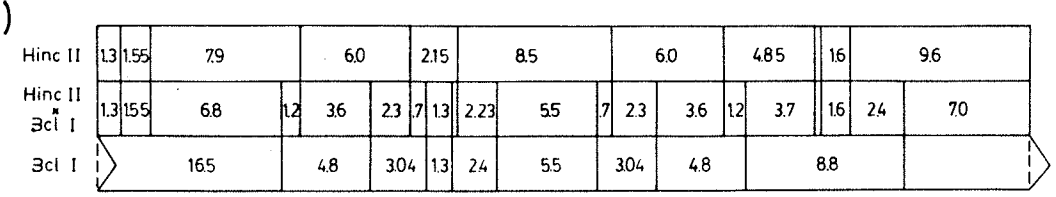
a)


Double Digest Fragment Generated By Overlap
Fragment
Orient.
20 16 12 8 4 0 4 8 12 16 20 kb
Fragment orient.
Predicted size(kb)
Observed size(kb)
Pvu II
Hinc II 3
A
4.1 7.55
Bcl I 1
B
10.5 6.4
A + B
6.81
6.85
Bcl I 1
C
6.4 10.5
A + C
7.96
Pst I Ava I Pst I
.53
Hinc II 4a,6,2,4b
X
5.65 2.2 8.7 5.65
Bcl I 5
Y
1.85 1.2 1.2 1.85
X + Y
2.38
2.32
Bcl I 5
Z
1.2 1.85 1.85 1.2
X + Z
1.73
Bcl I 6
M
.22 2.17
X + M
Bcl I 6
N
2.17 2.2
X + N
Pvu II Bam H I
Bam H I
Hinc II 3,4a,6,2,4b,5
R
7.45 6.18 2.2 8.7 6.18 4.74
Bcl I 4
S
3.75 1.07 1.07 3.75
R + S
.97
3.6
Bcl I 4
T
1.07 3.75 3.75 1.07
R + T
3.65
4 kb

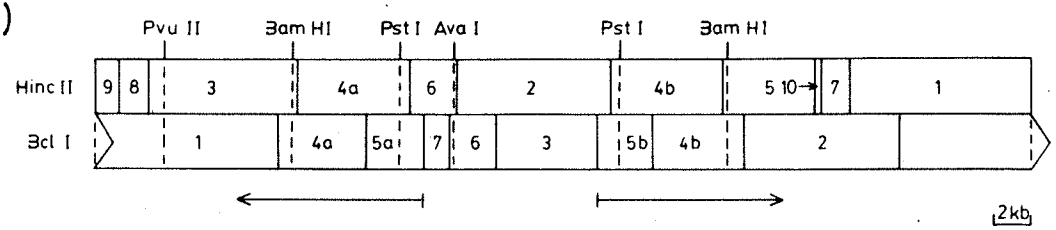
b)


Pvu II
Bam H I
Pst I Ava I
Pst I
Bam H I
Hinc II
9 8 3 4a 6 2 4b 5 10 7 1
Bcl I
1 4a 5a 6 5b 4b

c)


Hinc II
1.3 1.55 7.9 6.0 2.15 8.5 6.0 4.85 1.6 9.6
Hinc II
Bcl I
1.3 1.55 6.8 1.2 3.6 2.3 .7 1.3 2.23 5.5 .7 2.3 3.6 1.2 3.7 1.6 2.4 7.0
Bcl I
16.5 4.8 3.04 1.3 2.4 5.5 3.04 4.8 8.8

d)


Pvu II
Bam HI
Pst I Ava I
Pst I
Bam HI
Hinc II
9 8 3 4a 6 2 4b 5 10 7 1
Bcl I
1 4a 5a 7 6 3 5b 4b 2
2 kb

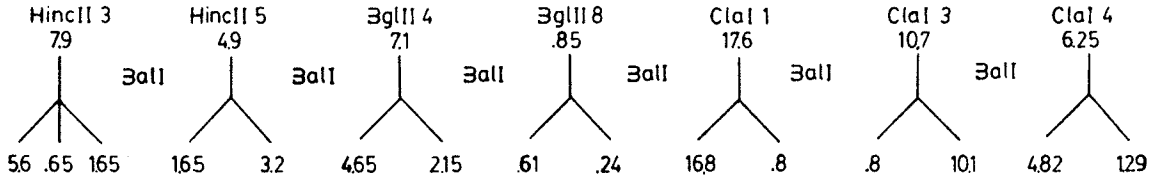
2.12 BalI restriction mapping

BalI cut A. klebsiana mtDNA three times and generated three bands that were present in equimolar amounts (Fig. 27B). The approximate sizes of the fragments were 27, 21, and 5.8 kb.

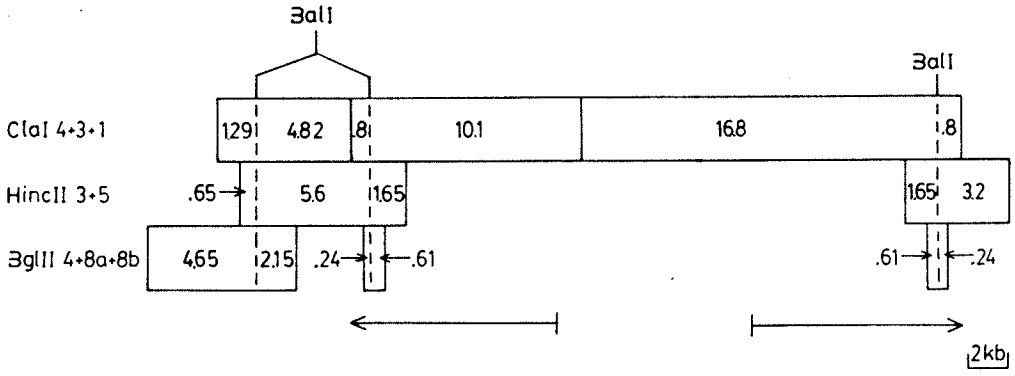
Codigestions of the mtDNA by various multisite enzymes with BalI showed that BalI cleaved the following fragments (see Fig. 9a); BglII4, BglII8a, and BglII8b (Fig. 27A, lane b), HincII3 and HincII5 (Fig. 27A, lane d), and ClaI1, ClaI3, and ClaI4 (Fig. 23B, lane c). Since the overlap between ClaI3 and HincII3 is within the repeat region, a BalI site must exist in each repeat, since BalI cleaves both fragments. The site can be located precisely using fragment sizes shown in Fig 9b. The third BalI site must be in the common sequence shared by ClaI4, HincII3, and BglII4. Precise location can again be deduced from fragment sizes shown. Based on the BalI codigest fragment sizes (Fig. 9a) and on the established map positions of ClaI, HincII, and BglII fragments, the three BalI sites were mapped as shown in Fig 9b.

Figure 9. Derivation of the BalI restriction map. a) Fragments HincII3, HincII5, BglII4, BglII8a, BglII8b, ClaI1, ClaI3, and ClaI4, were cleaved by BalI in the corresponding codigestions. The molecular sizes (kb) of the cleaved fragments and the codigest products are shown. The BalI cleavage products of fragments BglII8a and BglII8b were not detected on agarose gels after electrophoresis. b) Shown are the BalI sites on a partial restriction map consisting of the (previously mapped) fragments cleaved in codigestions with BalI. The sites were mapped by analysis of the codigest fragment sizes. Given the map positions of the BalI sites, the BalI cleavage products of fragments BglII8a and BglII8b are 0.61 and 0.24 kb fragments. The minimum size each inverted repeat region is shown by arrows.

a)


HincII 3
7.9
BalI
5.6 .65 1.65
HincII 5
4.9
BalI
1.65 3.2
BglII 4
7.1
BalI
4.65 2.15
BglII 8
.85
BalI
.61 .24
ClaI 1
17.6
BalI
16.8 .8
ClaI 3
10.7
BalI
.8 10.1
ClaI 4
6.25
4.82 1.29

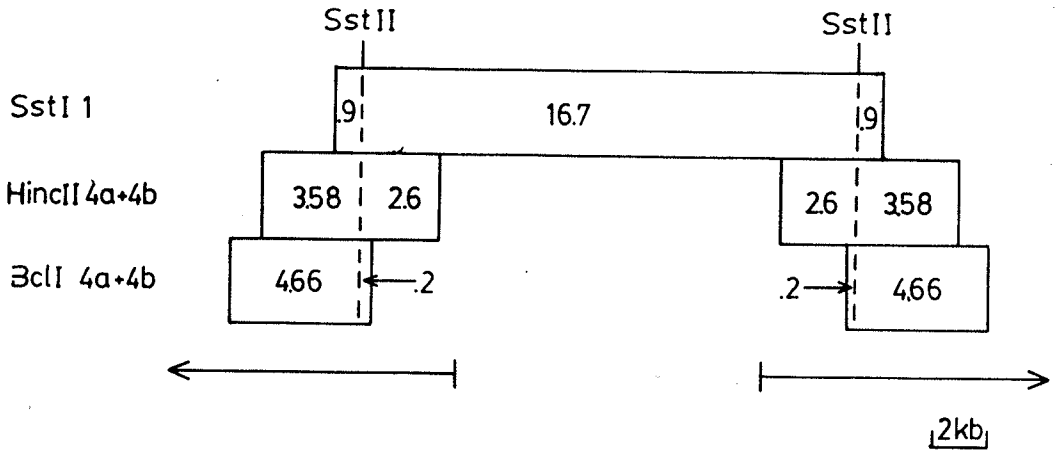
b)


BalI
BalI
ClaI 4+3+1
1.29
4.82
.8
10.1
16.8
.8
HincII 3+5
.65
5.6
1.65
1.65
3.2
BglII 4+8a+8b
4.65
2.15
.24
.61
.61
.24
2kb

2.13 SstII restriction mapping

Codigestions of A. klebsiana mtDNA by various multisite enzymes with SstII showed that SstII recognized the following fragments by cleaving them once each (see Table 4): HincII4a and HincII4b (Fig. 27C), and BstEII4a and BstEII4b (digest not shown). SstII also recognized SstI1 by cleaving it twice (digest not shown). Thus, the SstII codigestions indicated that there are two SstII sites in the mtDNA, one site in one repeat in the common sequence shared by SstI1, HincII4a, and BstEII4a, and the other site in the other repeat in the common sequence shared by SstI1, HincII4b, and BstEII4b. Based on the SstII codigest fragment sizes (Table 4) and on the established map positions of HincII, BstEII, and SstI fragments, the two SstII sites were mapped as shown in Fig 10.

Figure 10. Derivation of the SstII restriction map. Shown are the SstII sites on a partial restriction map consisting of the (previously mapped) fragments cleaved in codigestions by SstII with SstI, HincII, and BclI. The sites were mapped by analysis of the codigest fragment sizes. The minimum size of each inverted repeat region is shown by arrows.


SstII
SstII
SstI 1
.9
16.7
.9
HincII 4a+4b
3.58
2.6
2.6
3.58
BclI 4a+4b
4.66
.2
.2
4.66
2kb

2.14 Physical map of A. klebsiana mtDNA

A restriction map for A. klebsiana mtDNA was generated with 15 enzymes. Fig 11 summarizes the results of the mapping of these enzymes (Results sections 2.3-2.12). The mtDNA contains no sites for KpnI, XhoI, or XorII. It is obvious by the way the fragments overlap that the mtDNA is circular.
A circular restriction-site map is presented in Fig 12. The mean sizes of the site-to-site map distances from Fig 12 are listed in Table 5. The unit size of the A. klebsiana mt genome is taken to be 50.4 kb, as based on the sum of the fragment sizes (Table 5) of the restriction-site map (Fig. 12). This sum agrees closely with the value 50.3 $\pm$ 1.5 kb obtained as the mean of the sums of the products of single and double digestions (Results section 2.3).

It became apparent during restriction mapping that the mtDNA contained reiterated sequences in the form of inverted repeats (see Results sections 2.3-2.4). Examination of the restriction-site map (Fig. 12) shows that the two repeat regions are located at 15.3-34.5 and 77.7-96.8 m.u., respectively. The minimum length of each repeat (Fig. 12, solid areas ■) is 9.68 kb.
A region of uncertainty is indicated between the last repeated site and the nearest non-repeated site, which could extend the repeat areas to 12.45 kb (Fig. 12, open areas □).

Figure 11. Summary of the derivation of the restriction map of A. klebsiana mtDNA. The arrows show the minimum ■, and maximum □, sizes of the inverted repeat regions.

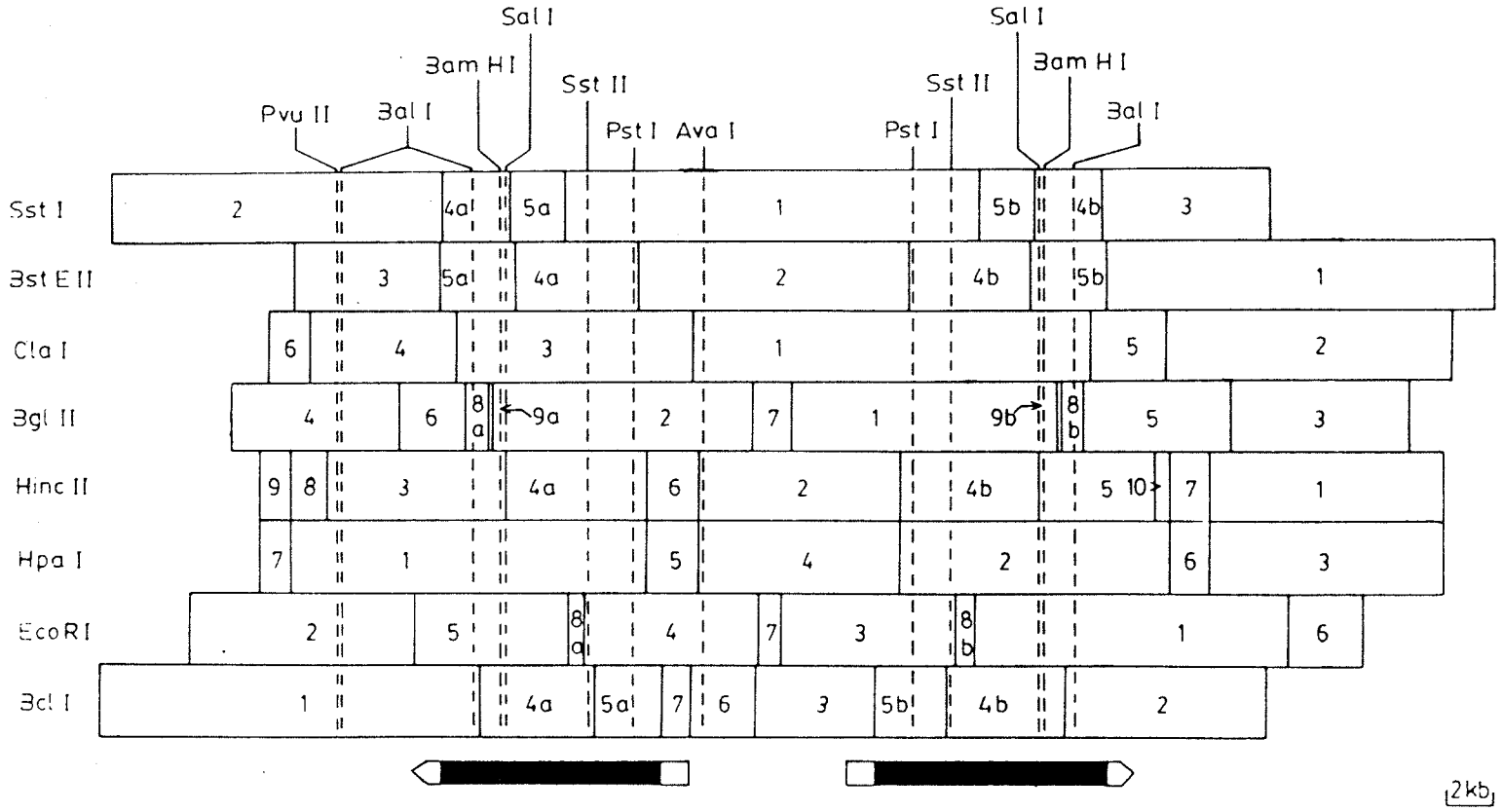

Sal I
Bam HI
Sst II
Pvu II
Bal I
Pst I
Ava I
Sst I
Bst EII
Cla I
Bgl II
Hinc II
Hpa I
EcoRI
Bcl I
2kb

Figure 12. Restriction-site map of A. klebsiana mtDNA. Minimum ■, and maximum □, limits of the inverted repeat regions. The arrows indicate the midpoints between the inverted repeat regions, and the centers of flip-flop rotation. The map is calibrated in both map units (m.u.) and kilobase pairs (kb). The unique AvaI site in the small unique region is taken to be 0 m.u./0 kb. A map unit is defined as 1% of the genome (1 m.u. = 0.504 kb).

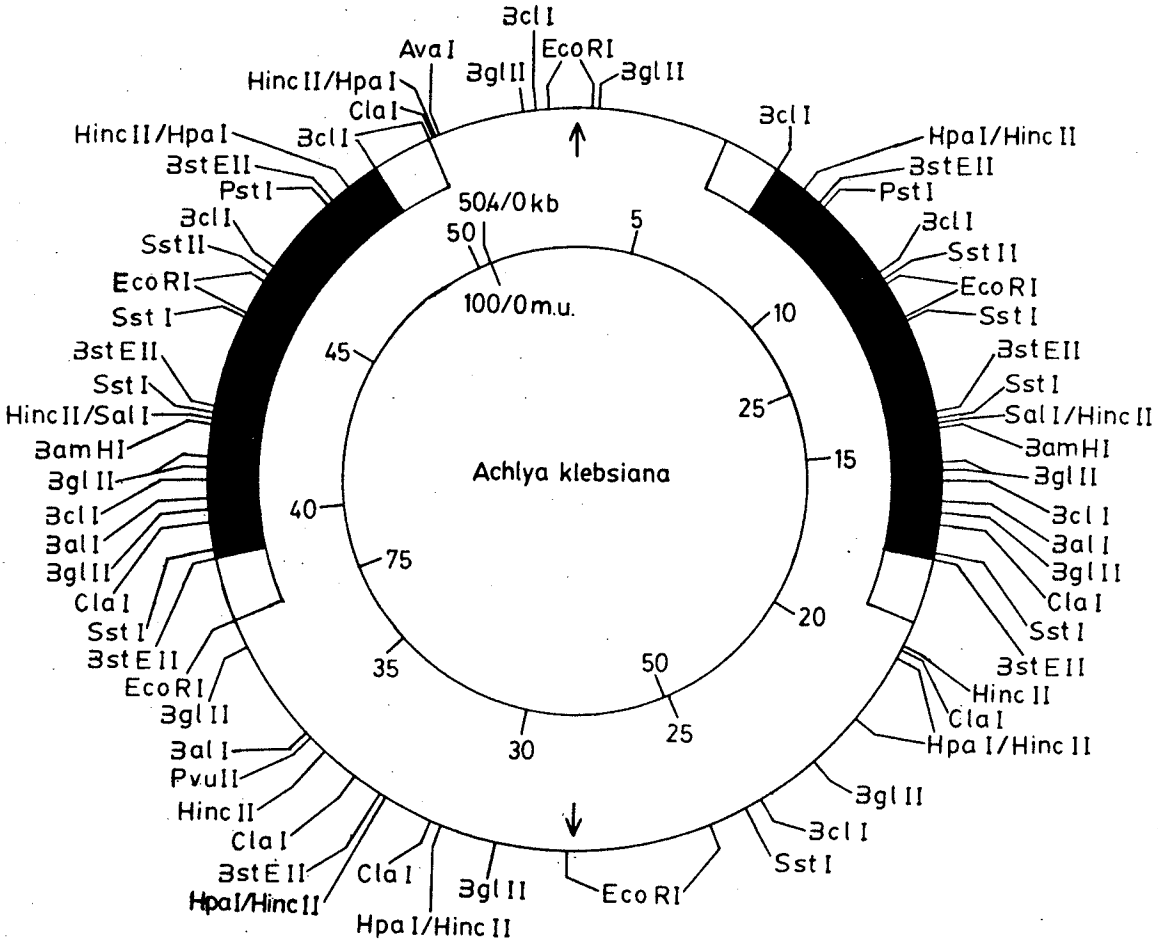

Achlya klebsiana
504/0 kb
100/0 m.u.
5
10
15
20
25
30
35
40
45
50
75
BclI
EcoRI
BglII
AvaI
HincII/HpaI
ClaI
BstEII
PstI
SstII
SstI
HpaI/HincII
SalI/HincII
BamHI
BalI
PvuII

Table 5. Mean sizes of the site-to-site map fragments from the A. klebsiana mitochondrial genome.

Fragment	Size (kb)	Position (m.u.)	Fragment	Size (kb)	Position (m.u.)
AvaI-BglII	1.90	0-3.8	BglII-HpaI/HincII	1.30	59.8-62.4
BglII-BclI	0.29	3.8-4.3	HpaI/HincII-ClaI	0.22	62.4-62.8
BclI-EcoRI*	0.30	4.3-4.9	ClaI-Hpa/HincII	1.10	62.8-65.0
EcoRI-EcoRI	0.95	4.9-6.8	HpaI/HincII-BstEII	0.10	65.0-65.2
EcoRI-BglII*	0.15	6.8-7.1	BstEII-ClaI	0.65	65.2-66.5
BglII-BclI	4.16	7.1-15.3	ClaI-HincII	0.85	66.5-68.2
BclI-HpaI/HincII	0.72	15.3-16.8	HincII-PvuII	0.45	68.2-69.0
HpaI/HincII-BstEII	0.48	16.8-17.7	PvuII-BalI*	0.15	69.0-69.3
BstEII-PstI*	0.10	17.7-17.9	BalI-BglII	2.25	69.3-73.8
PstI-BclI	1.85	17.9-21.6	BglII-EcoRI*	0.65	73.8-75.1
BclI-SstII	0.20	21.6-22.0	EcoRI-BstEII*	1.30	75.1-77.7
SstII-EcoRI*	0.10	22.0-22.2	BstEII-SstI	0.10	77.7-77.9
EcoRI-EcoRI	0.87	22.2-23.9	SstI-ClaI	0.60	77.9-79.0
EcoRI-SstI*	0.06	23.9-24.0	ClaI-BglII	0.30	79.0-79.6
SstI-BstEII	2.15	24.0-28.3	BglII-BalI*	0.25	79.6-80.1
BstEII-SstI	0.20	28.3-28.7	BalI-BclI*	0.40	80.1-80.9
SstI-SalI/HincII*	0.05	28.7-28.8	BclI-BglII	0.21	80.9-81.3
SalI/HincII-BamHI	0.10	28.8-29.0	BglII-BglII	0.20	81.3-81.7
BamHI-BglII	0.74	29.0-30.4	BglII-BamHI	0.74	81.7-83.2
BglII-BglII	0.20	30.4-30.8	BamHI-SalI/HincII	0.10	83.2-83.4
BglII-BclI	0.21	30.8-31.2	SalI/HincII-SstI*	0.05	83.4-83.5
BclI-BalI*	0.40	31.2-32.0	SstI-BstEII	0.20	83.5-83.9
BalI-BglII*	0.25	32.0-32.5	BstEII-SstI	2.15	83.9-88.2
BglII-ClaI	0.30	32.5-33.1	SstI-EcoRI*	0.06	88.2-88.3
ClaI-SstI	0.60	33.1-34.3	EcoRI-EcoRI	0.87	88.3-90.0
SstI-BstEII	0.10	34.3-34.5	EcoRI-SstII*	0.10	90.0-90.2
BstEII-HincII	2.00	34.5-38.5	SstII-BclI	0.20	90.2-90.6
HincII-ClaI*	0.10	38.5-38.7	BclI-PstI	1.85	90.6-94.3
ClaI-HpaI/HincII*	0.20	38.7-39.1	PstI-BstEII*	0.10	94.3-94.5
HpaI/HincII-HpaI/HincII	1.61	39.1-42.3	BstEII-HpaI/HincII	0.48	94.5-95.4
HpaI/HincII-BglII	1.30	42.3-44.9	HpaI/HincII-BclI	0.72	95.4-96.8
BglII-BclI	1.42	44.9-47.7	BclI-BclI	1.32	96.8-99.4
BclI-SstI*	0.40	47.7-48.5	BclI-ClaI*	0.15	99.4-99.7
SstI-EcoRI*	0.90	48.5-50.3	ClaI-HpaI/HincII*	0.03	99.7-99.8
EcoRI-EcoRI	3.19	50.3-56.6	HpaI/HincII-AvaI*	0.05	99.8-100
EcoRI-BglII	1.60	56.6-59.8		50.40	

Fragments are listed in sequential order starting from the O site (AvaI) as represented in Figure 12. Map fragments were estimated from double digests unless otherwise indicated. Restriction enzyme fragment sizes generated from these map coordinates were within 10% of experimental values.

A map unit (m.u.) is 1% of the genome size (504 bp).

* Size estimated from the restriction fragment map (Figure 11).

2.15 Presence of both orientations of the non-repeated regions

Intramolecular homologous recombination between inverted repeats can reverse the orientation of the included unique regions so that a population of DNA molecules may contain orientational or flip-flop isomers (Adelburg and Berquist, 1972; Guerineau et al, 1976). The presence of flip-flop isomers can be tested for by appropriate restriction digests. Any fragment that extends from a repeat to a unique region will be unchanged in either orientation of the unique region. This is the case when *A. klebsiana* mtDNA is digested by *BalI* (Fig. 27B), which has a single site in each repeat region and a single site in the large unique region (Fig. 12). Three bands are generated whose sizes sum to one genome length regardless of flip-flop rotation (Table 6). However, fragments that originate in one unique region and terminate in the other unique region will be sensitive to changes in orientation. This was tested for in *A. klebsiana* by an *AvaI*/*PvuII* codigestion of the mtDNA (Fig. 13). Four bands were generated whose molecular sizes sum to twice the genome length (Table 6). Thus, the *AvaI*/*PvuII* codigestion indicates that both possible orientations of the *A. klebsiana* mt genome are present in the mtDNA population.

Figure 13. Restriction digests of A. klebsiana mtDNA to visualize orientational isomers. Both lanes are AvaI/PvuII digests: a) digestion for 6 h, and b) digestion for 17 h. Electrophoresis was for 20 h at 30 V through a 0.4% agarose gel.

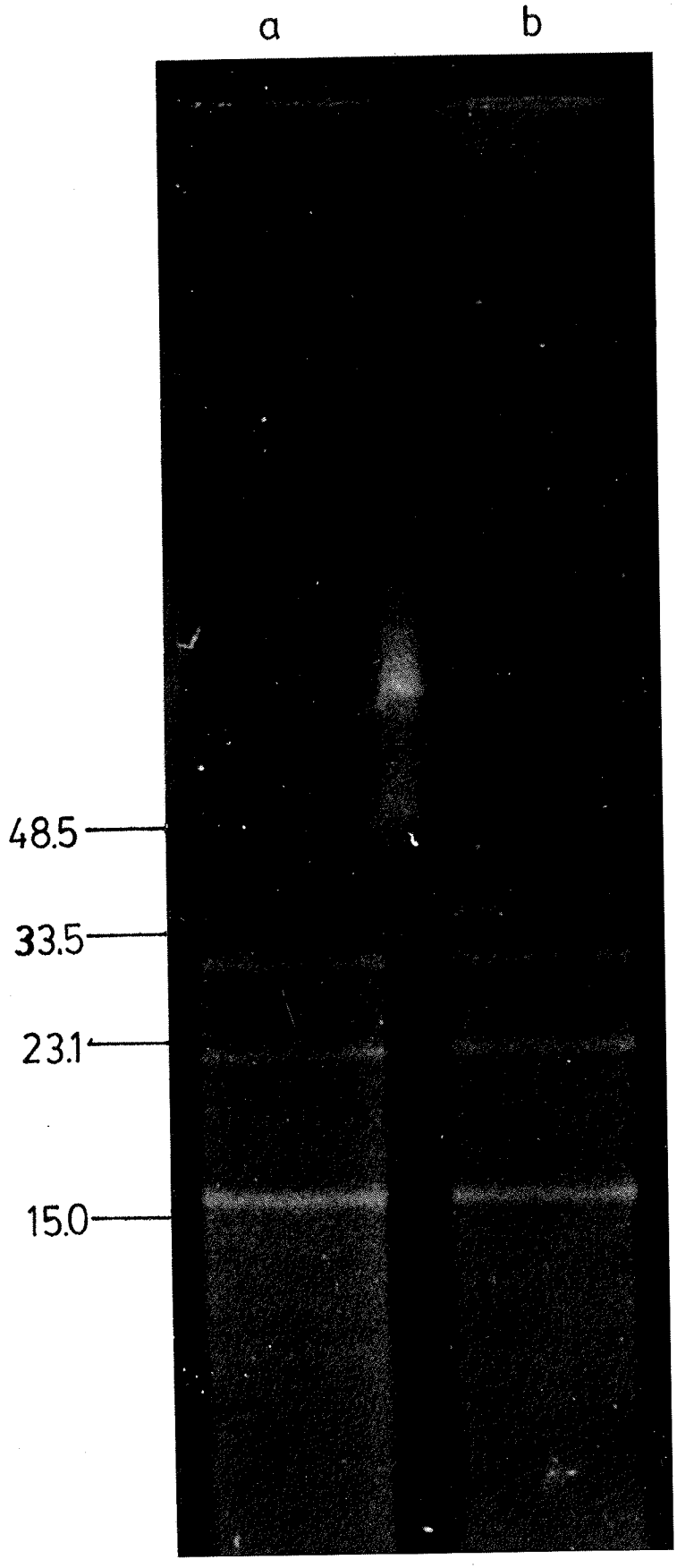

a
b
48.5
33.5
23.1
15.0

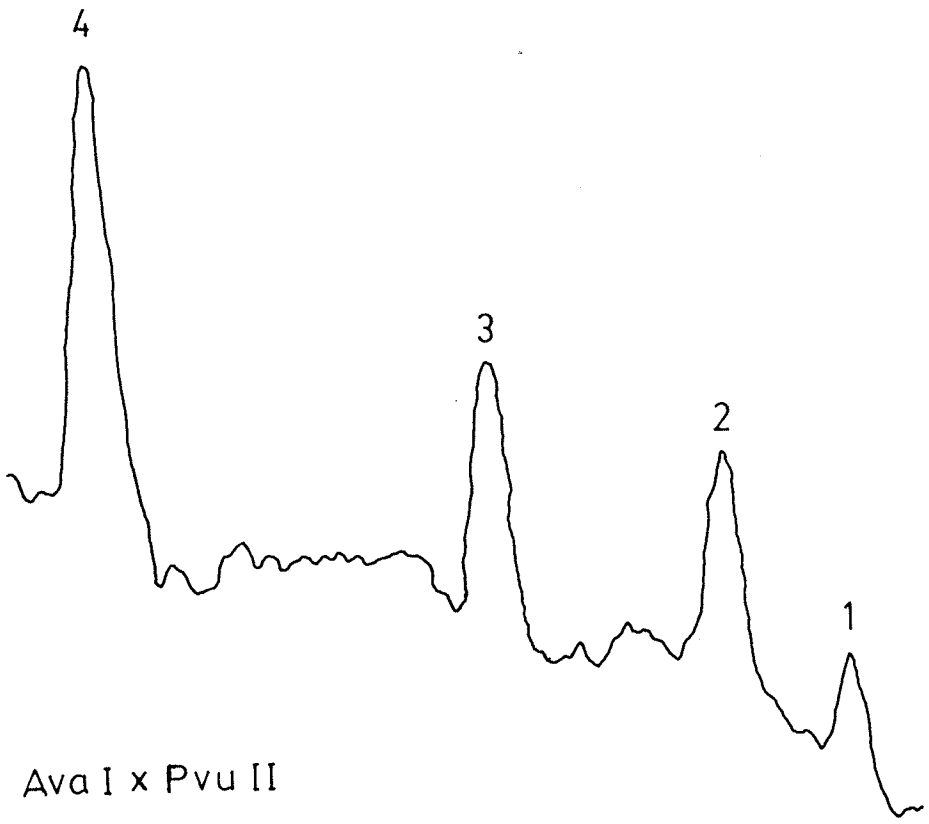
Table 6. Restriction digest showing both orientations of the *A. klebsiana* mitochondrial genome.

	Fragment size (kb)				Fragment sums		
Digest	1	2	3	4	1-4	1+4	2+3
BalI	27.0	20.0	5.8		52.8		
AvaI X PvuII	35.8	30.0	22.5	16.4	104.7	52.2	52.5

It follows that the center of rotation for each unique region coincides with the midpoint of that region (Fig. 12, arrows). The restriction-site map (Fig. 12) thus arbitrarily represents one of its two possible orientations.

A densitometer scan of the bands in Fig 13 indicates that the amount of DNA in bands 1 plus 4 is roughly equal to the amount in bands 2 plus 3 (Fig. 14). Thus, it is concluded that the two orientational isomers are present in the mtDNA population in approximately equimolar amounts.

Figure 14. Densitometric scan of a photograph of ethidium bromide fluorescence in the bands of the AvaI/PvuII codigestion of A. klebsiana mtDNA shown in Fig 13a.



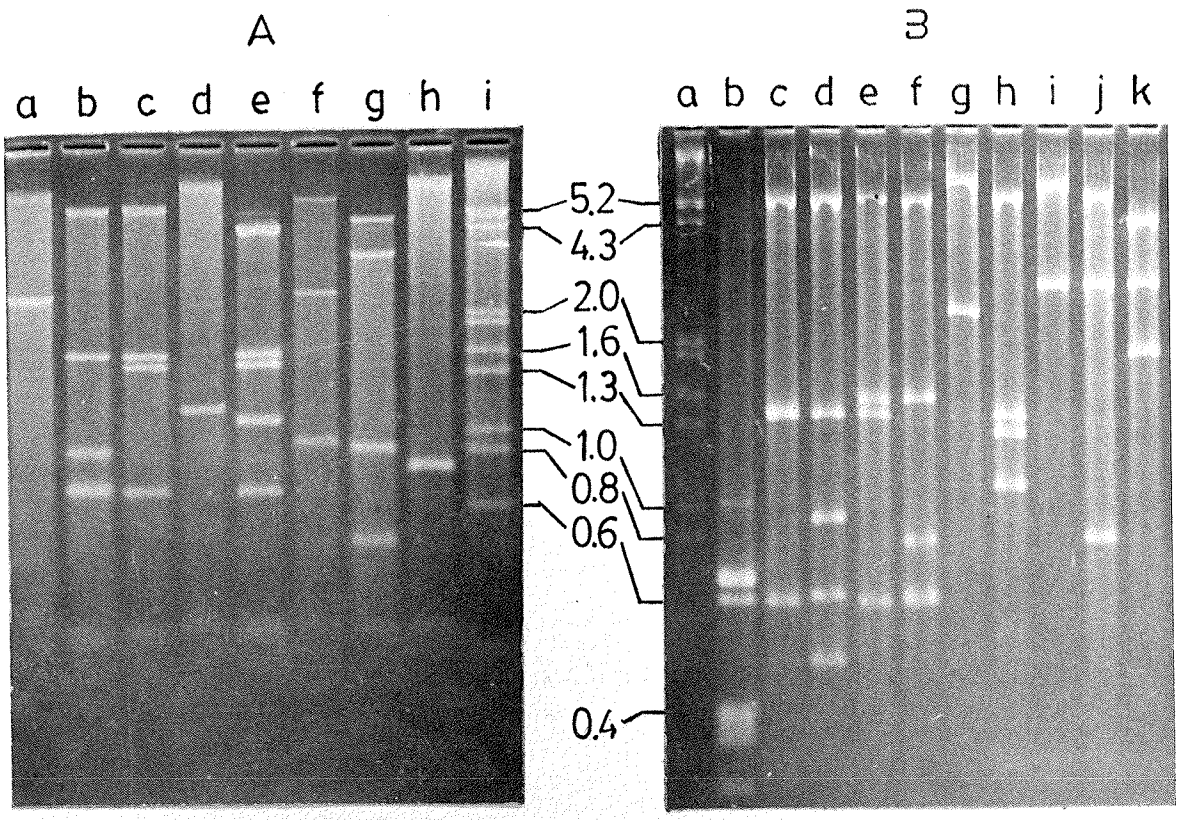
Ava I x Pvu II

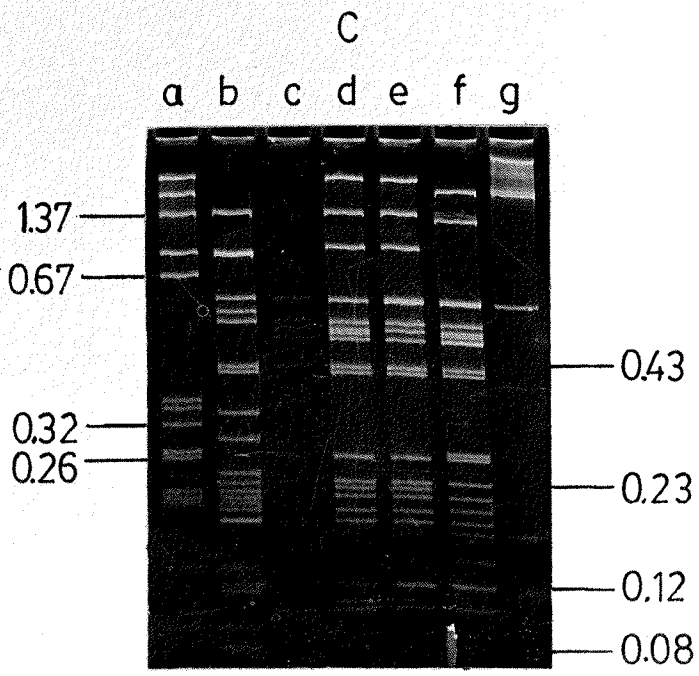
3. Cloning of A. klebsiana mtDNA

3.1 Cloning into pBR322

A. klebsiana mtDNA was digested with BclI and cloning of fragments into the BamHI site of pBR322 was attempted as described in Materials and Methods section 4.7. The plasmids from 8 ampicillin-resistant, tetracycline-sensitive colonies were isolated and run unrestricted on an agarose gel (data not shown). Preliminary size estimates indicated that the inserts were significantly larger than BclI6 (2.42 kb), which contains the unique AvaI site of the mtDNA. The unique AvaI site in pBR322 could then be used to linearize the plasmids and to determine an accurate molecular size. In three sucessful AvaI restrictions (data not shown) the linearized plasmids were found to be 9.2-9.3 kb in size. Since pBR322 has a size of 4.36 kb, insert size was approximately 4.84-4.94 kb. This matches with BclI4, whose size was determined to be 4.89 kb (Table 1). To determine whether in fact BclI4 had been cloned, one of the recombinant plasmids (designated pMAK5) was subjected to restriction analysis with enzymes whose sites had been mapped in A. klebsiana mtDNA. The insert map was found to match the BclI4 map perfectly (compare Fig. 16 with Fig. 12). Fig 15 shows some examples of pMAK5 restriction digests. BglII digests of pMAK5 run on polyacrylamide gels (data not shown) showed that there was an additional BglII site in BclI4 that was

Figure 15. Digests of pMAK5 with various restriction enzymes. A) Digests produced SstI (lane a), SstI/HindIII (lane b), HindIII (lane c), BglII/BstEII (lane d), BglII/HindIII (lane e), BglII/SstI (lane f), BglII/HincII (lane g), and BglII/BamHI (lane h). Electrophoresis was for 5 h at 90 V through a 1.2% agarose gel. The size scale is based on a HindIII/EcoRI digest of lambda DNA (lane i). B) Digests produced by HindIII/BstEII (lane c), HindIII/EcoRV (lane d), HindIII (lane e), HindIII/SstI (lane f), SstI (lane g), SstI/EcoRV (lane h), EcoRV (lane i), EcoRV/BstEII (lane j), and EcoRV/BglII (lane k). Electrophoresis was for 19 h at 50 V through a 1.5% agarose gel. The size scale is based on a HindIII/EcoRI digest of lambda DNA (lane a), and a NciI digest of pBR322 DNA (lane b). C) Digests produced by Sau3A (lane a), Sau3A/HaeIII (lane b), HaeIII/EcoRV (lane d), HaeIII (lane e), HaeIII/HindIII (lane f), and HindIII (lane g). Electrophoresis was for 7 h at 80 V through a 6.0% polyacrylamide gel. The size scale is based on a HaeIII digest of pBR322 (lane c), and on Sau3A fragments of pMAK5 DNA that originated wholly from the pBR322 part of the plasmid (lane a).


A
a b c d e f g h i
B
a b c d e f g h i j k
5.2
4.3
2.0
1.6
1.3
1.0
0.8
0.6
0.4


C
a b c d e f g
1.37
0.67
0.32
0.26
0.43
0.23
0.12
0.08

not initially mapped for the mtDNA (see Results section 2.6). Fig 16 shows the restriction-site map of pMAK5. In addition to sites already reported for BclI4, sites for AvaII, EcoRV, HaeIII, HpaII, HindIII, NciI, Sau3A, and TaqI were added by intensive mapping of the insert using agarose and polyacrylamide gels.

To further show that the insert from pMAK5 originated from A. klebsiana mtDNA and was BclI4, the plasmid was radioactively labelled and used as a probe against a Southern blot of some mtDNA restriction digests (Fig. 17). The hybridization results were as predicted from the mtDNA restriction map (see Figs. 11 and 12), thus confirming that the insert in pMAK5 did originate from the A. klebsiana mt genome.

Figure 16. Restriction-site map of plasmid pMAK5. Plasmid pMAK5 was constructed by cloning the BclI4 fragment of A. klebsiana mtDNA into the BamHI site of pBR322. Restriction sites mapped for pMAK5 that were not mapped for A. klebsiana mtDNA are those for AvaII, EcoRV, HaeIII, HpaII, HindIII, NciI, Sau3A, and TaqI. The known sites for AvaII, HaeIII, HpaII, NciI, Sau3A, and TaqI, are not shown for the pBR322 part of the plasmid. The gene for ampicillinase (Amp^R) and the region of the origin of replication (ori) are shown.

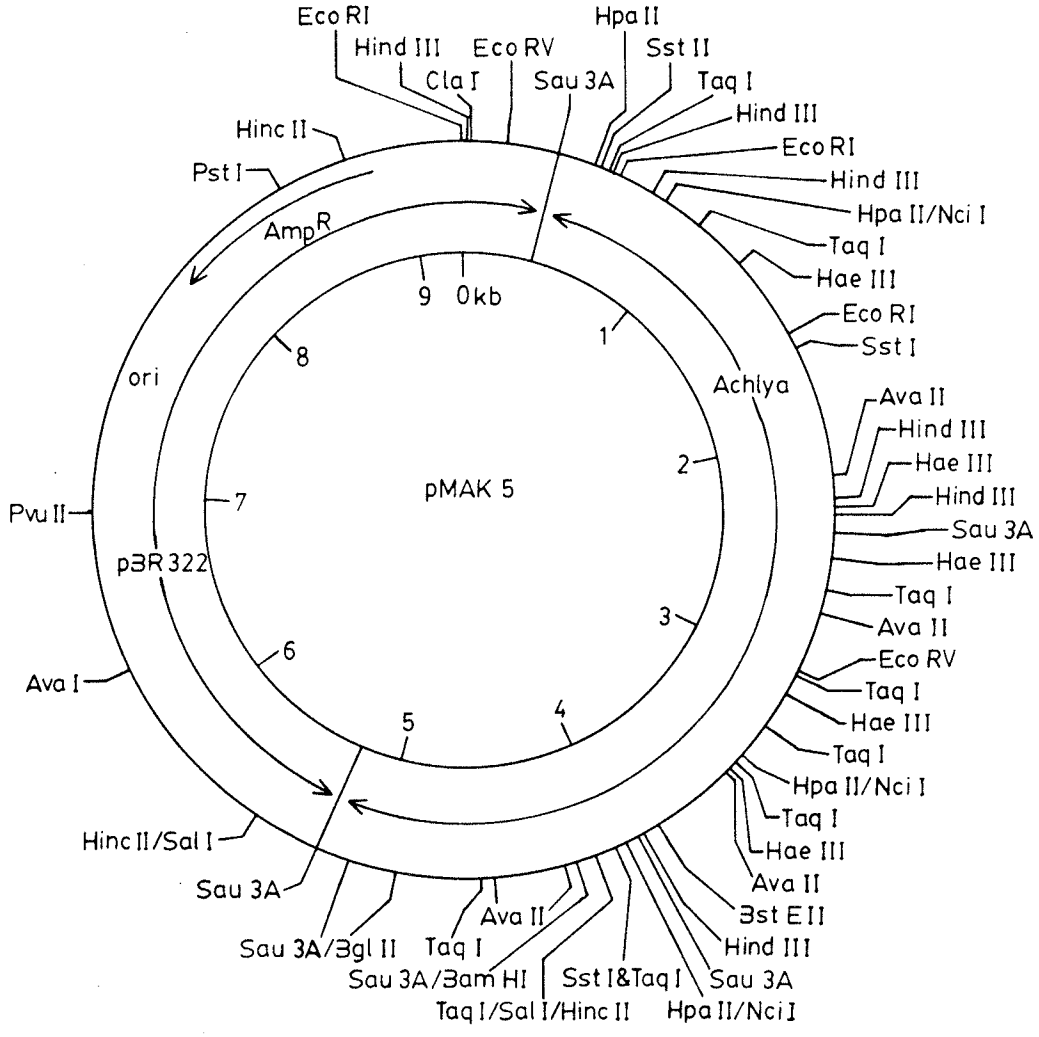
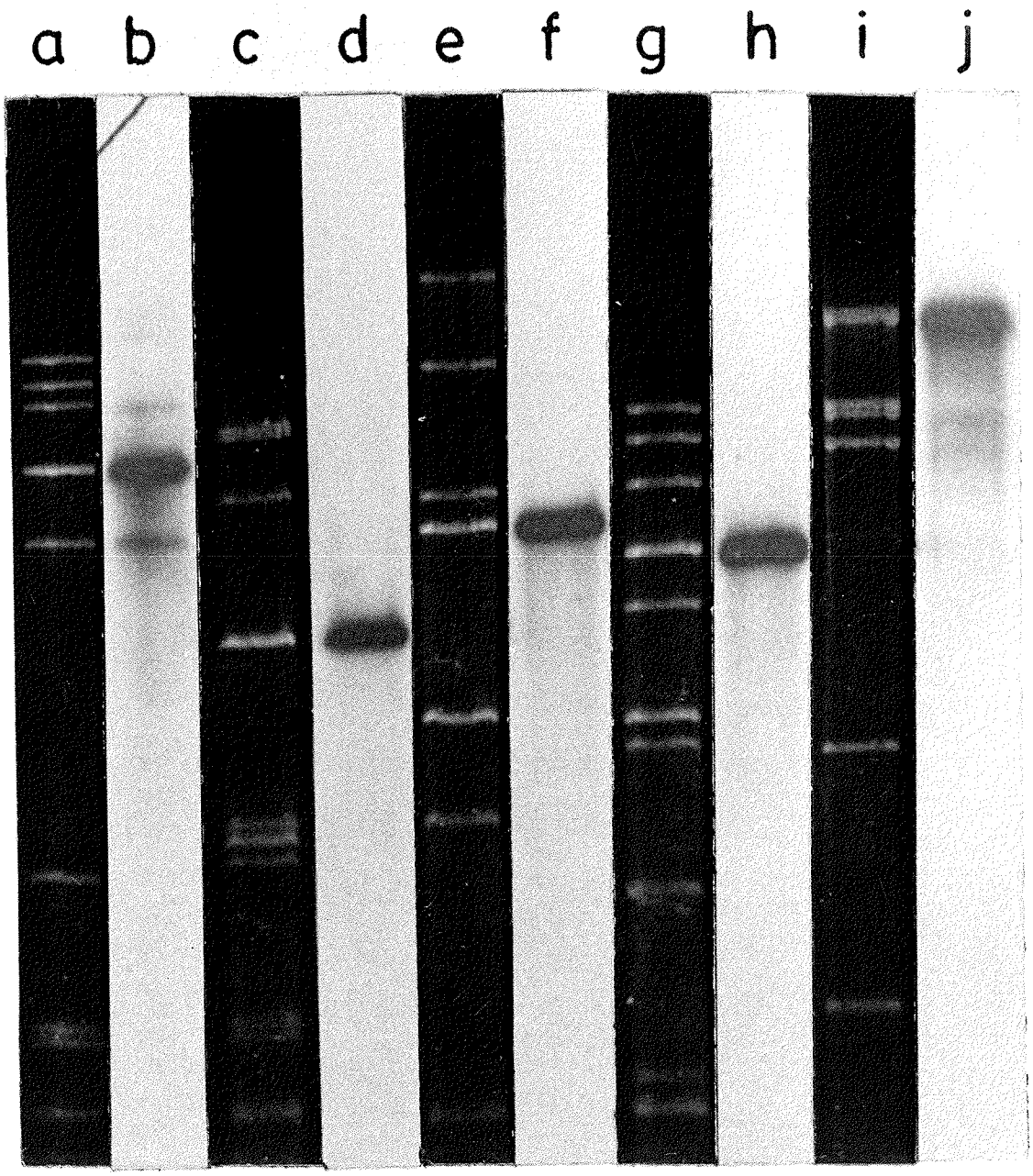

Eco RI
Hind III
Cla I
Eco RV
Sau 3A
Hpa II
Sst II
Taq I
Hind III
Eco RI
Hind III
Hpa II/Nci I
Taq I
Hae III
Eco RI
Sst I
Ava II
Hind III
Hae III
Hind III
Sau 3A
Hae III
Taq I
Ava II
Eco RV
Taq I
Hae III
Taq I
Hpa II/Nci I
Taq I
Hae III
Ava II
Bst E II
Hind III
Sau 3A
Hpa II/Nci I
Sst I&Taq I
Taq I/Sal I/Hinc II
Sau 3A/Bam HI
Taq I
Ava II
Sau 3A/Bgl II
Sau 3A
Hinc II/Sal I
Ava I
Pvu II
Hinc II
Pst I
AmpR
ori
pBR 322
pMAK 5
Achlya
0kb
1
2
3
4
5
6
7
8
9

Figure 17. Blot transfer hybridization of A. klebsiana mtDNA restriction digests with ^{32}P-labelled pMAK5 DNA. a) HincII digest; b) blot transfer hybridization of a; c) BclI/HincII digest; d) blot transfer hybridization of c; e) BclI digest; f) blot transfer hybridization of e; g) BclI/BglII digest; h) blot transfer hybridization of i. Electrophoresis was for 14.5 h at 35 V through 0.8% agarose gel. Autoradiography was for 1 h at 22°C.


a b c d e f g h i j

3.2 Cloning into pUC9

A. klebsiana mtDNA was digested with EcoRI and cloning of the fragments into the EcoRI site of pUC9 was attempted as described in Materials and Methods section 4.8. The plasmids from 35 ampicillin resistant, white colonies were isolated, restricted with EcoRI, and the inserts sized (data not shown). Thirty-four of the plasmids contained inserts whose sizes were not equal to any of the mtDNA EcoRI fragments and no further analysis was done on them. One plasmid had an insert whose size (6.5 kb) indicated that it could be EcoRI5. To determine whether in fact EcoRI5 had been cloned, the recombinant plasmid containing the 6.5 kb insert (designated pDAB6.5) was subjected to restriction analysis with enzymes whose sites had been mapped in A. klebsiana mtDNA. The insert map was found to match the EcoRI5 map perfectly (compare Fig. 19 with Fig. 12). Fig 18 shows some examples of pDAB6.5 restriction digests. In the EcoRI/BclI and EcoRI/ClaI codigestions (Fig. 18, lanes f and h) it can be seen that BclI and ClaI were inhibited. This was probably due to methylation of the internal adenine in their recognition sites by the dam methylase of the host cell (see Discussion). Fig 19 shows the restriction-site map of pDAB6.5. In addition to sites already reported for EcoRI5, sites for EcoRV and HindIII were added by mapping of the insert using agarose gels.

Figure 18. Digests of pDAB6.5 and pMAK5 with various restriction enzymes. A) Digests of pDAB6.5 produced by EcoRI (lane b), EcoRI/SstI (lane c), EcoRI/BstEII (lane d), EcoRI/HincII (lane e), EcoRI/BclI (lane f), EcoRI/BglII (lane g), and EcoRI/ClaI (lane h). Electrophoresis was for 17 h at 65 V through a 0.85% agarose gel. The size scale is based on HindIII (lane a), and HindIII/EcoRI (lane i), digests of lambda DNA. B) Digests of pDAB6.5 produced by EcoRI/SstI (lane d), EcoRI/SstI/EcoRV (lane e), EcoRI/EcoRV (lane f), EcoRI/HindIII/EcoRV (lane g), EcoRI/HindIII (lane h), and EcoRI (lane i), and digests of pMAK5 produced by SstI (lane b), SstI/EcoRV (lane c), HindIII/EcoRV (lane j), and HindIII (lane k). Electrophoresis was for 18 h at 65 V through a 1% agarose gel. The size scale is based on HindIII (lane m), and HindIII/EcoRI (lane a and l) digests of lambda DNA, and EcoRI digests of pUC9 DNA (2.7 kb band in lanes a and l).

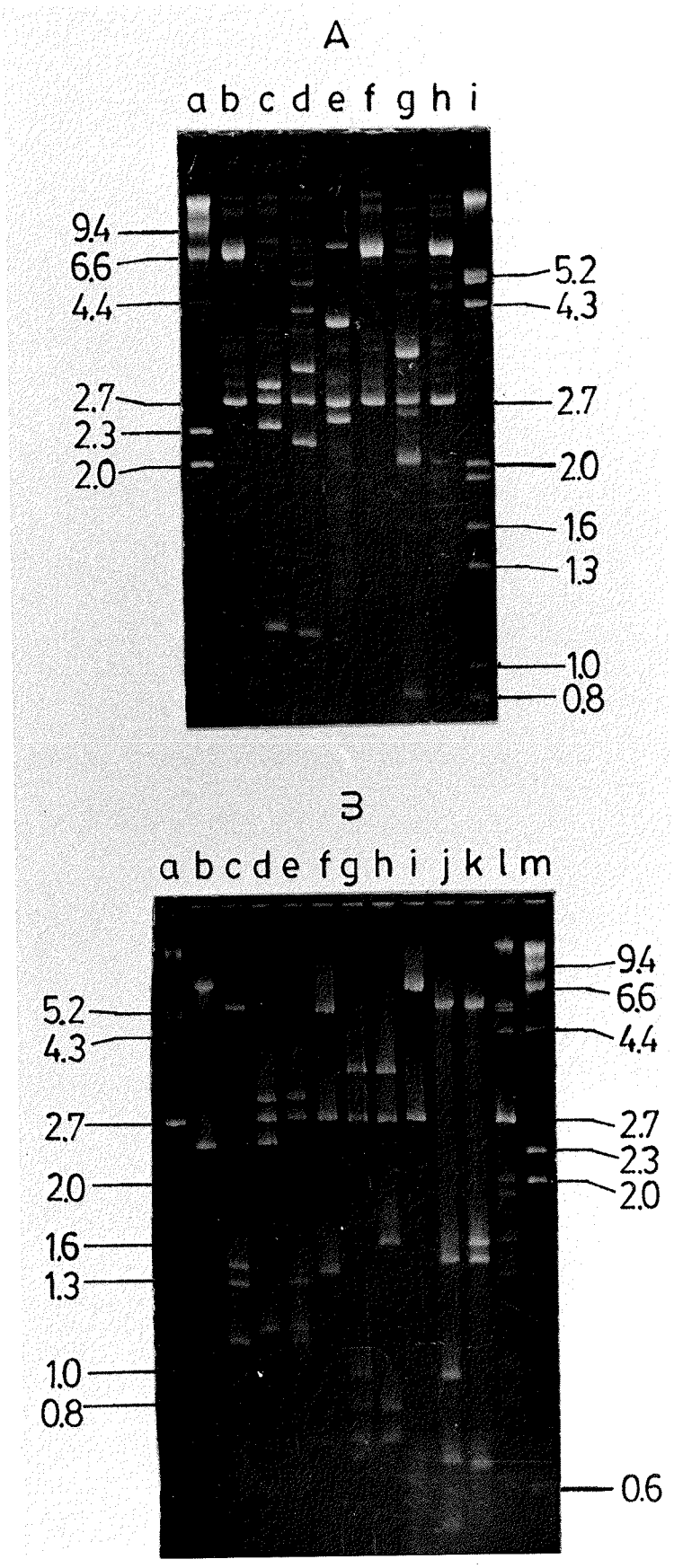
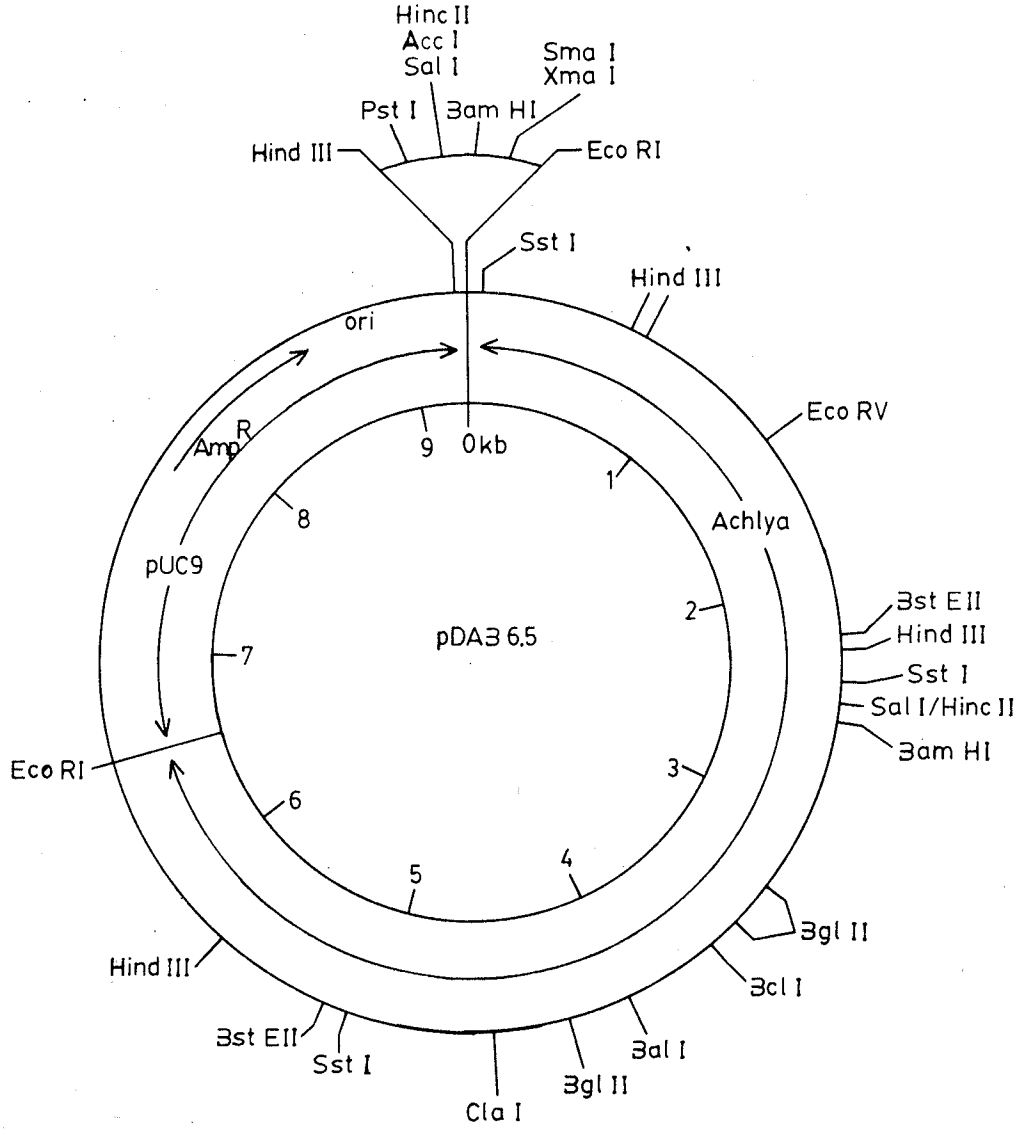

A
a b c d e f g h i
9.4
6.6
4.4
2.7
2.3
2.0
5.2
4.3
2.7
2.0
1.6
1.3
1.0
0.8
B
a b c d e f g h i j k l m
5.2
4.3
2.7
2.0
1.6
1.3
1.0
0.8
9.4
6.6
4.4
2.7
2.3
2.0
0.6

Figure 19. Restriction-site map of plasmid pDAB6.5. Plasmid pDAB6.5 was constructed by cloning the EcoRI5 fragment of A. klebsiana mtDNA into the EcoRI site of pUC9. Restriction sites mapped for pDAB6.5 that were not mapped for A. klebsiana mtDNA are those for EcoRV and HindIII. The gene for ampicillinase (Amp^R) and the region of the origin of replication (ori) are shown.


Hinc II
Acc I
Sal I
Sma I
Xma I
Pst I
Bam HI
Hind III
Eco RI
Sst I
Hind III
ori
Eco RV
Amp R
9
0kb
1
8
Achlya
pUC9
2
Bst EII
Hind III
Sst I
Sal I/Hinc II
Bam HI
pDAB 6.5
7
Eco RI
3
6
4
5
Bgl II
Bcl I
Hind III
Bst EII
Sst I
Bal I
Bgl II
Cla I

To further show that the insert from pDAB6.5 originated from A. klebsiana mtDNA and was EcoRI5, the plasmid was radioactively labelled and used as a probe against a Southern blot of some mtDNA restriction digests (Fig. 20). The hybridization results were as predicted from the mtDNA restriction map (see Figs. 11 and 12), thus confirming the insert from pDAB6.5 did originate from the A. klebsiana mt genome.

Fig 21 shows the regions of the A. klebsiana mt genome from which the inserts in pMAK5 and pDAB6.5 originate. The insert from pMAK5 could have originated from either repeat region, i.e. can be either BclI4a or BclI4b. It can also be seen in Fig 21 that the cloned fragments, BclI4 and EcoRI5, share approximately 3.7 kb of common sequence. The two recombinant plasmids therefore share some common restriction fragments, as is shown in Fig 18B. Fig 21 also shows that EcoRI5 contains the region between the last repeated restriction site mapped (a BstEII site at 77.7 m.u. as represented in Fig. 12) and the nearest non-repeated site in the large unique region (an EcoRI site, defining one end of EcoRI5, at 75.1 m.u. as represented in Fig. 12). Thus, pDAB6.5 contains the region (about 1.3 kb) in which the junction between the repeat regions and the large unique region lies.

Figure 20. Blot transfer hybridization of A. klebsiana mtDNA restriction digests with ^{32}P-labelled pDAB6.5 DNA. a) BglII digest; b) blot transfer hybridization of a; c) ClaI digest; d) blot transfer hybridization of c; e) EcoRI digest; f) blot transfer hybridization of e; g) HincII digest; h) blot transfer hybridization of g. Electrophoresis was for 18 h at 55 V through a 0.7% agarose gel. Autoradiography was for 8 h at 22°C.

a b c d e f g h

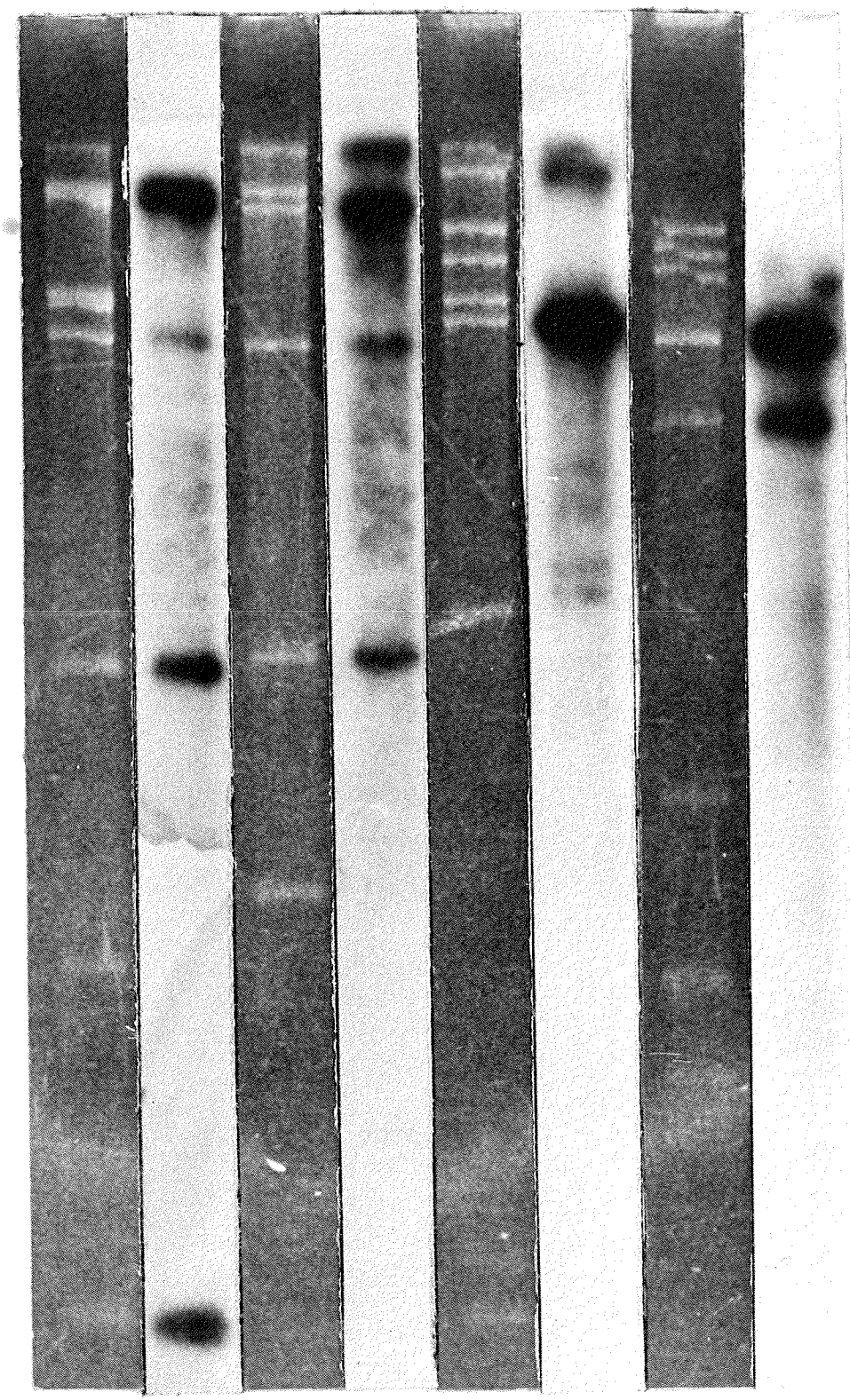
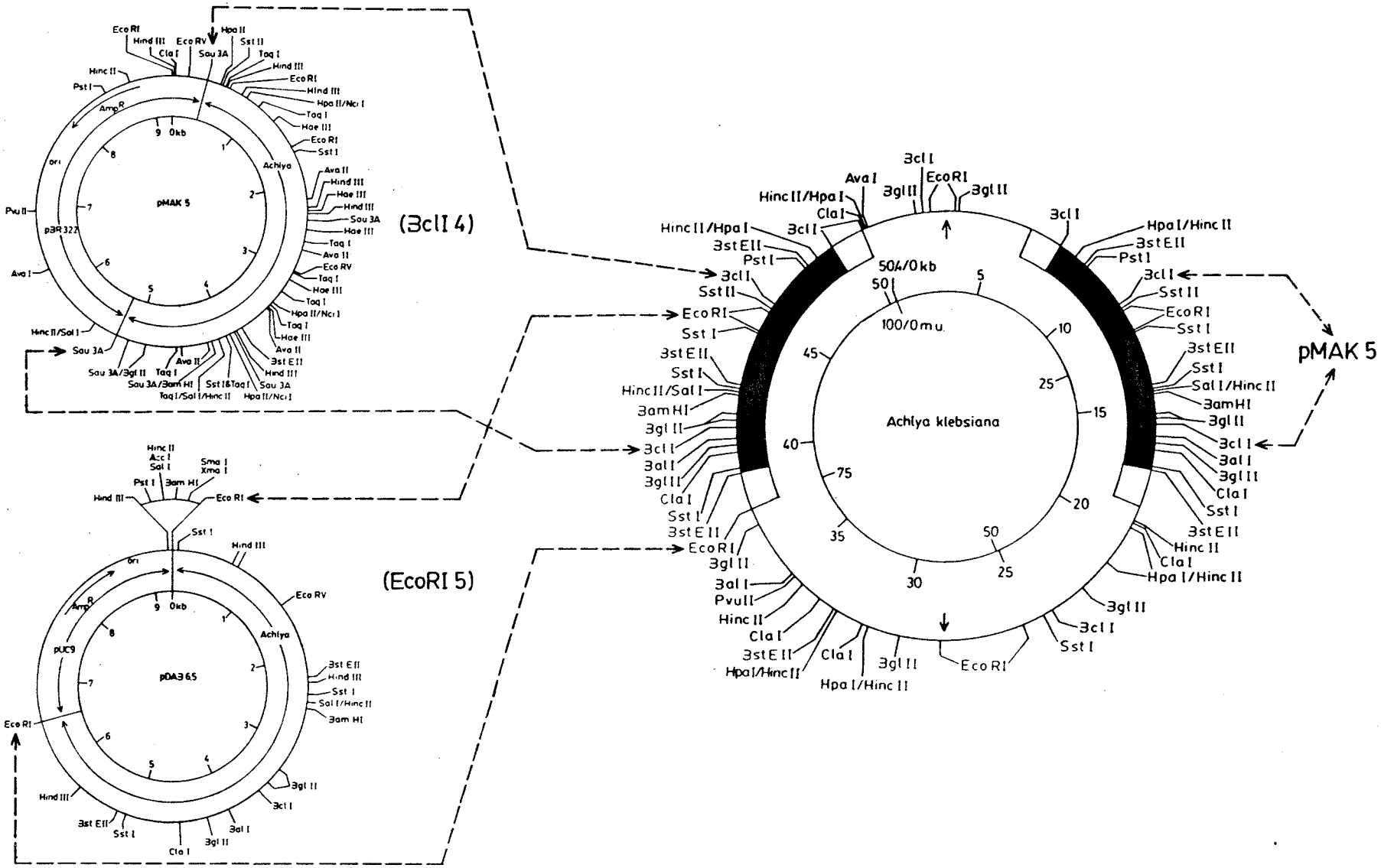


Figure 21. Regions of the A. klebsiana mt genome from which the Achlya sequences of pMAK5 (Bcl4) and pDAB6.5 (EcoRI5) originate. Plasmid pMAK5 could contain the BclI4 fragment from either repeat region (i.e. either BclI4a or BclI4b).


pMAK 5
(BclI 4)
(EcoRI 5)
Achlya klebsiana
504/0kb
100/0 m.u.
pBR322
pUC9
pDA365
AmpR
ori

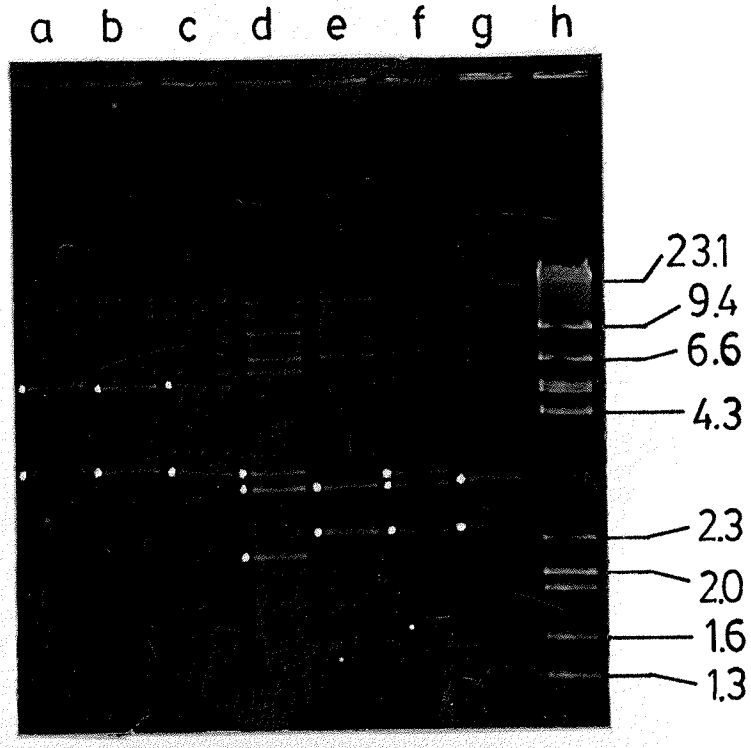
4. Results Appendix

To avoid duplication of gel photographs in various parts of the text and to avoid excessive paste-up procedures, gel photographs are grouped in this section (Figs. 22-27) and referred to in the text where appropriate.

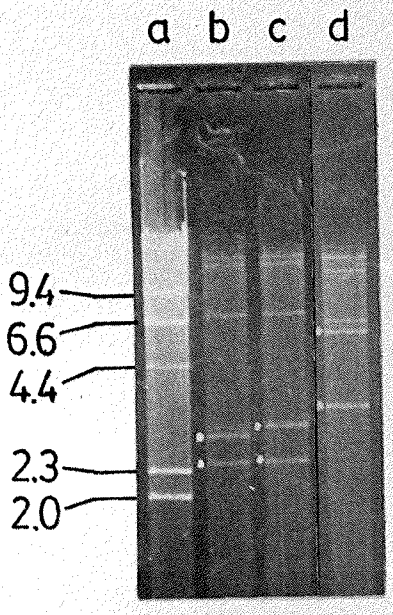
Figures 22-27. Fragments of A. klebsiana mtDNA generated by single and multiple digestions with restriction enzymes. Digests were electrophoresed through agarose gels and the gels stained with ethidium bromide before being photographed under ultraviolet light (see Materials and Methods section 3). White circles indicate DNA in greater than molar amounts (see Results section 2.2). The size scales are given in kilobase pairs and are based on HindIII and/or HindIII/EcoRI and/or EcoRI digests of lambda DNA and/or unrestricted lambda DNA.

Figure 22. A) Digests produced by BstEII/AvaI (lane a), BstEII/PstI (lane b), BstEII (lane c), BstEII/SstI (lane d), SstI (lane e), SstI/PstI (lane f), and SstI/AvaI (lane g). Electrophoresis was for 4 h at 90 V through a 0.8% agarose gel. The BstEII digest (lane c) shows some large partial digestion products. The size scale was based on HindIII and HindIII/EcoRI digests of lambda DNA (lane h). B) Digests produced by SstI/BamHI (lane b), SstI (lane c), and BstEII (lane d). Electrophoresis was for 5 h at 90 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a). C) Digests produced by BclI (lane b), BstEII (lane c), and BstEII/AvaI (lane d). Electrophoresis was for 5 h at 80 V through a 0.8% agarose gel. The size scale was based on HindIII (lane a) and EcoRI (lane e) digests of lambda DNA.

A


a b c d e f g h
23.1
9.4
6.6
4.3
2.3
2.0
1.6
1.3

B


a b c d
9.4
6.6
4.4
2.3
2.0

C

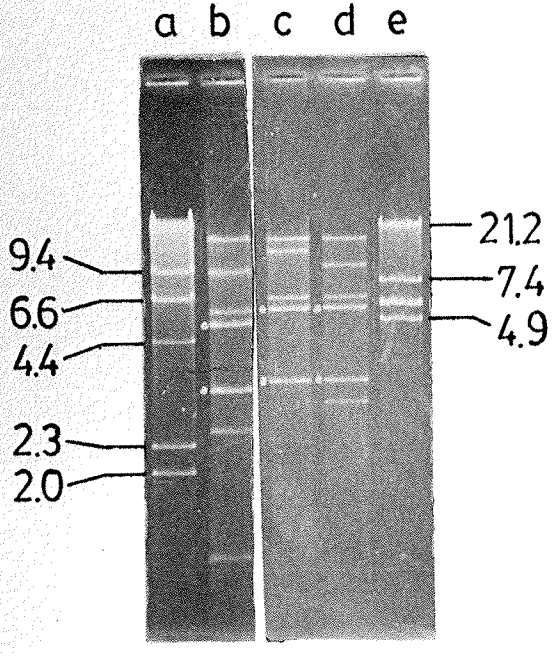
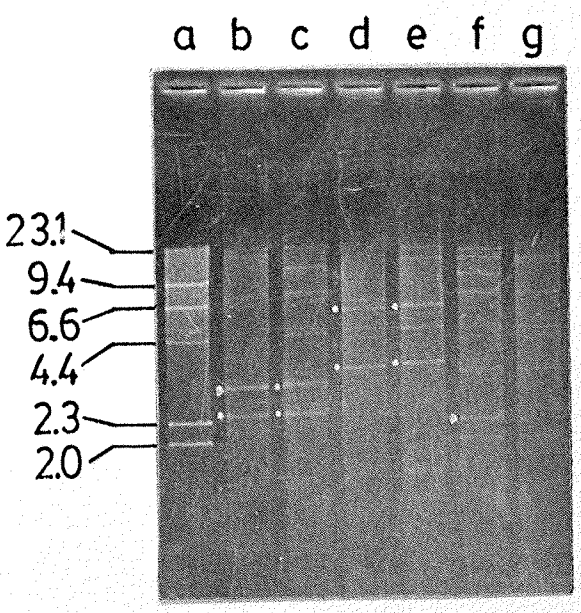
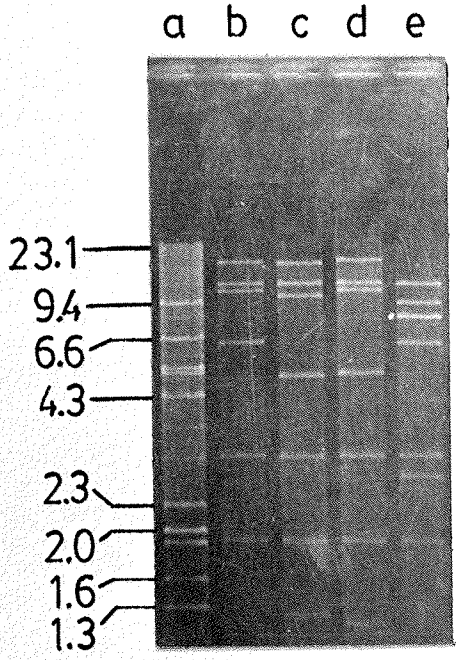

a b c d e
9.4
6.6
4.4
2.3
2.0
21.2
7.4
4.9

Figure 23. A) Digests produced by SstI (lane b), SstI/PvuII (lane c), BstEII (lane d), BstEII/PvuII (lane e), ClaI/BamHI (lane f), and ClaI (lane g). Electrophoresis was for 4 h at 100 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a). B) Digests produced by ClaI (lane b), ClaI/BalI (lane c), ClaI/PvuII (lane d), and ClaI/PstI (lane e). Electrophoresis was for 5 h at 80 V through a 0.6% agarose gel. The size scale was based on HindIII and HindIII/EcoRI digests of lambda DNA (lane a). C) Digests produced by BglII (lane b), BglII/BstEII (lane c), BstEII (lane d), BclI/BstEII (lane e), BclI (lane f), SstI (lane g), SstI/ClaI (lane h), and ClaI (lane i). Electrophoresis was for 5.5 h at 75 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a).

A


a b c d e f g
23.1
9.4
6.6
4.4
2.3
2.0

B


a b c d e
23.1
9.4
6.6
4.3
2.3
2.0
1.6
1.3

C

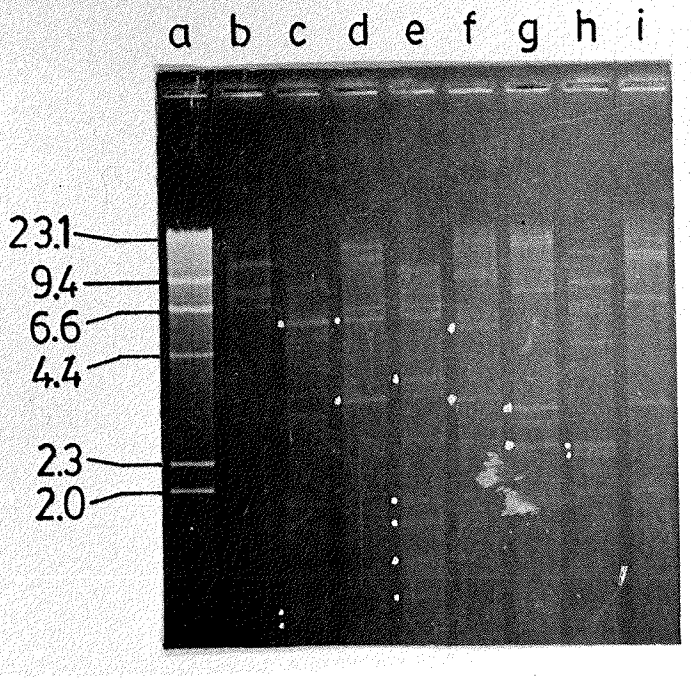
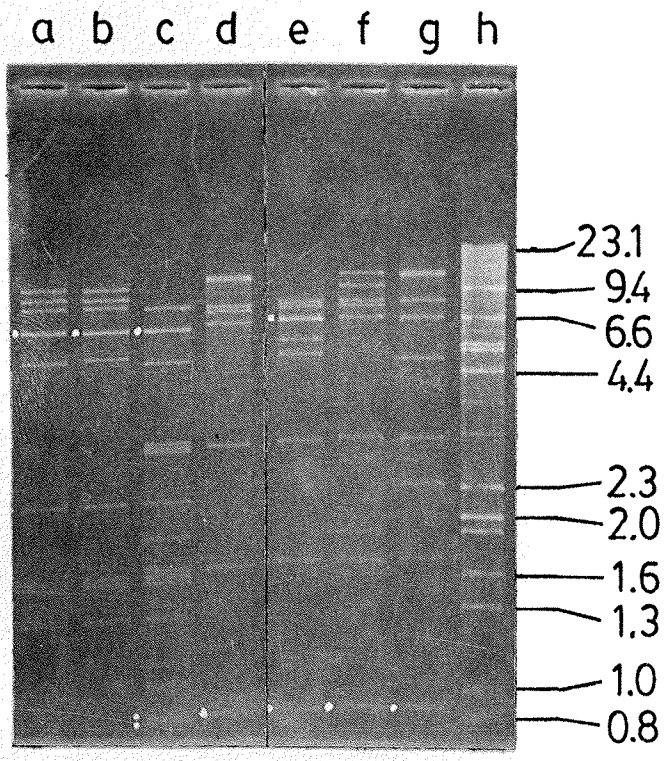

a b c d e f g h i
23.1
9.4
6.6
4.4
2.3
2.0

Figure 24. A) Digests produced by HincII/BamHI (lane a), HincII (lane b), BglII/HincII (lane c), BglII (lane d), BglII/PstI (lane e), BglII/AvaI (lane f), and BglII/PvuII (lane g). Electrophoresis was for 5.5 h at 90 V through a 0.8% agarose gel. The size scale was based on HindIII and HindIII/EcoRI digests of lambda DNA (lane h). B) Digests produced by BglII/ClaI (lane a), BglII/BamHI (lane b), BglII (lane c), BglII/HincII (lane d), BglII/HincII/PvuII (lane e), HincII (lane f), and HincII/ClaI (lane g). Electrophoresis was for 5 h at 95 V through a 0.7% agarose gel. The size scale was based on HindIII (lane h) and HindIII/EcoRI (lane i) digests of lambda DNA.

A


a b c d e f g h
23.1
9.4
6.6
4.4
2.3
2.0
1.6
1.3
1.0
0.8

B

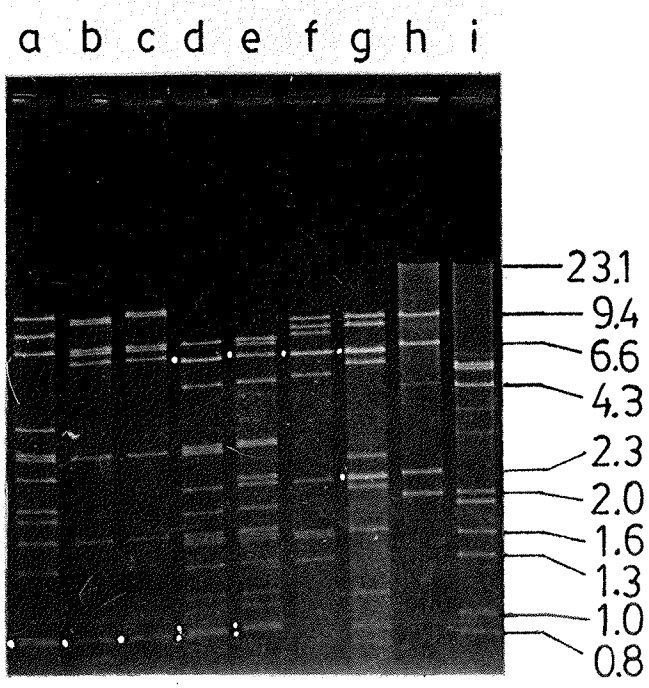

a b c d e f g h i
23.1
9.4
6.6
4.3
2.3
2.0
1.6
1.3
1.0
0.8

Figure 25. A) Digests produced by ClaI (lane a), ClaI/HincII (lane b), and HincII (lane c). Electrophoresis was for 6 h at 80 V through a 0.8% agarose gel. The size scale was based on HindIII and HindIII/EcoRI digests of lambda DNA (lane d). B) Digests produced by EcoRI (lane b), EcoRI/HincII (lane c), HincII (lane d), HincII/PstI (lane e), and HincII/PvuII (lane f). Electrophoresis was for 6 h at 80 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a). C) Digests produced by HincII/AvaI (lane b), HincII (lane c), HpaI (lane d), HpaI/PstI (lane e), and HpaI/PvuII (lane f). Electrophoresis was for 4.5 h at 75 V through a 0.65% agarose gel. The HpaI/PvuII digest shows some large partial digestion products. The size scale was based on a HindIII digest of lambda DNA (lane a). D) Digests produced by BclI (lane a), BclI/HincII (lane b), HincII (lane c), HincII/SstI (lane d), and SstI (lane e). Electrophoresis was for 4 h at 80 V through a 0.8% agarose gel. The BclI and BclI/HincII digests show some large partial digestion products. A 3.7 kb fragment is nearly indistinguishable from the 3.6 kb double band in the BclI/HincII digest. The 2.35 kb fragment in the HincII/SstI digest is a triplet consisting of a double band and a similar-sized non-homologous fragment. The size scale was based on a HindIII digest of lambda DNA (lane f).

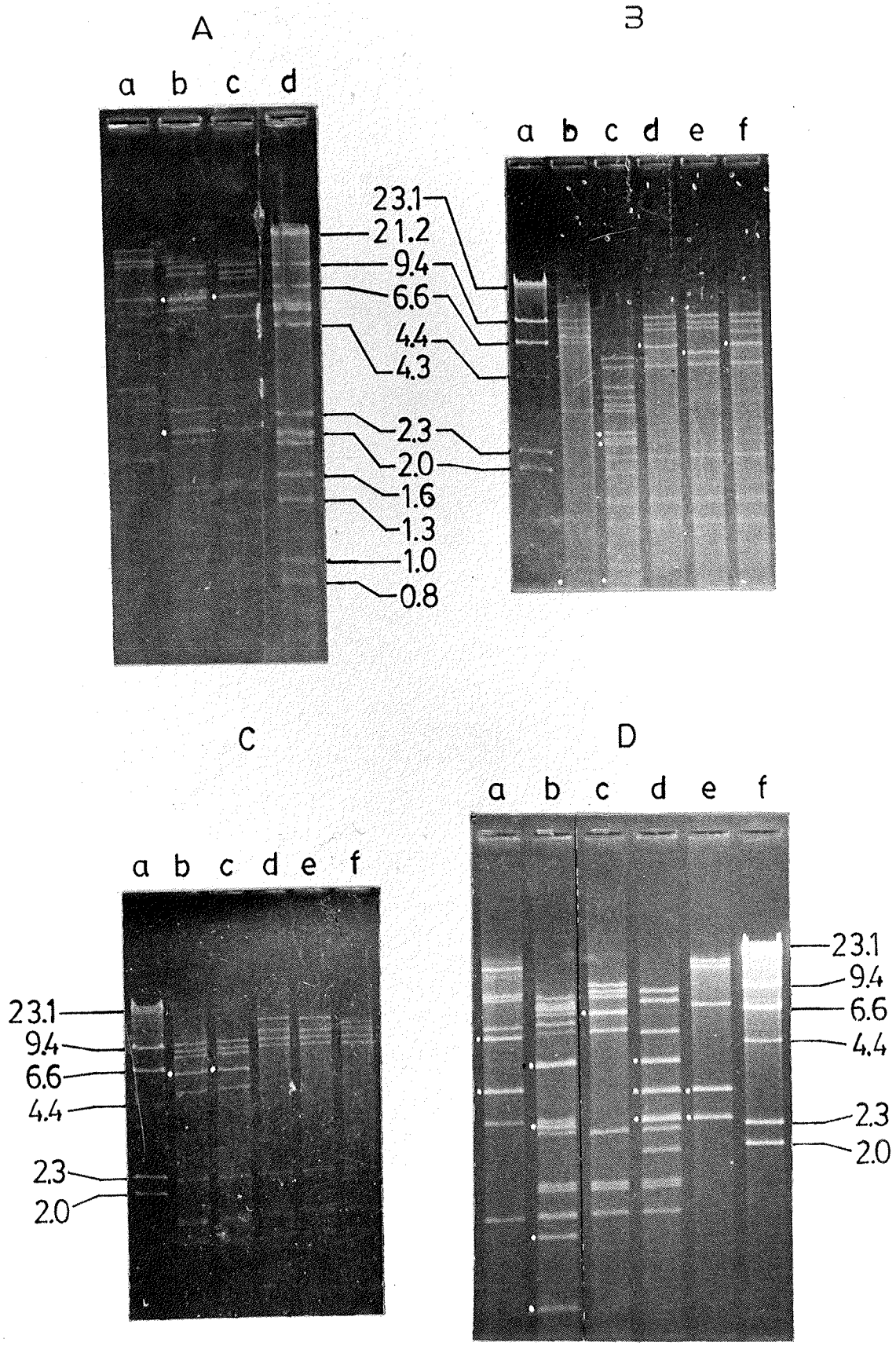

A
a b c d
23.1
21.2
9.4
6.6
4.4
4.3
2.3
2.0
1.6
1.3
1.0
0.8
B
a b c d e f
C
a b c d e f
23.1
9.4
6.6
4.4
2.3
2.0
D
a b c d e f
23.1
9.4
6.6
4.4
2.3
2.0

Figure 26. A) Digests produced by EcoRI/SalI (lane b), EcoRI/BamHI (lane c), EcoRI (lane d), EcoRI/HpaI (lane e), HpaI (lane f), and HpaI/BamHI (lane g). Electrophoresis was for 14.25 h at 35 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a). B) Digests produced by HpaI (lane b), and HpaI/SalI (lane c). Electrophoresis was for 12 h at 35 V through a 0.7% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a). C) Digests produced by HincII (lane a), BclI/HincII (lane b), BclI (lane c), BclI/BglII (lane d), and BglII (lane e). Electrophoresis was for 14.5 h at 35 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane f). D) Digests produced by BclI (lane b), BclI/PvuII (lane c), BclI/AvaI (lane d), BclI/PstI (lane e), and BclI/BamHI (lane f). Electrophoresis was for 14.5 h at 40 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a).

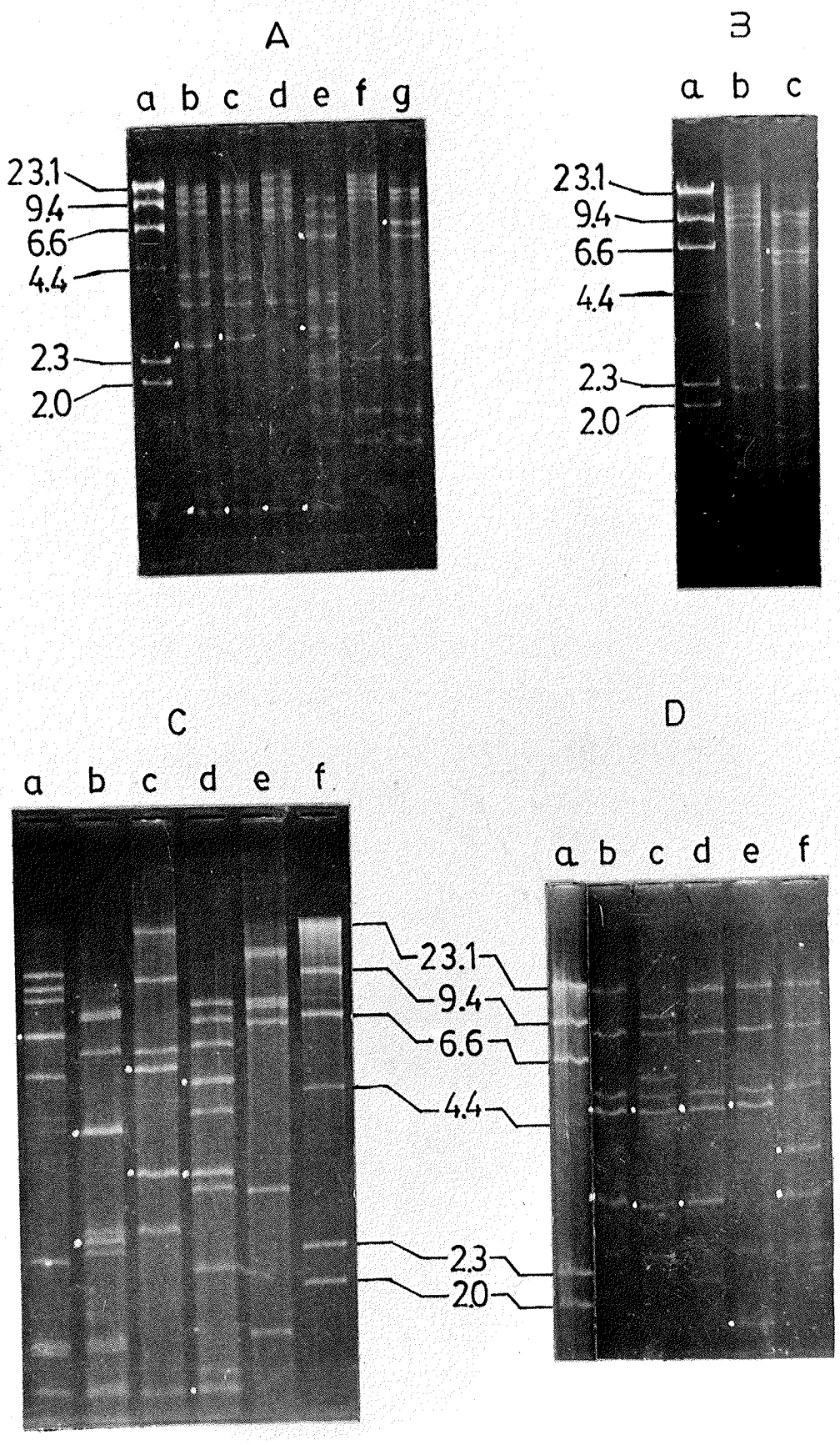
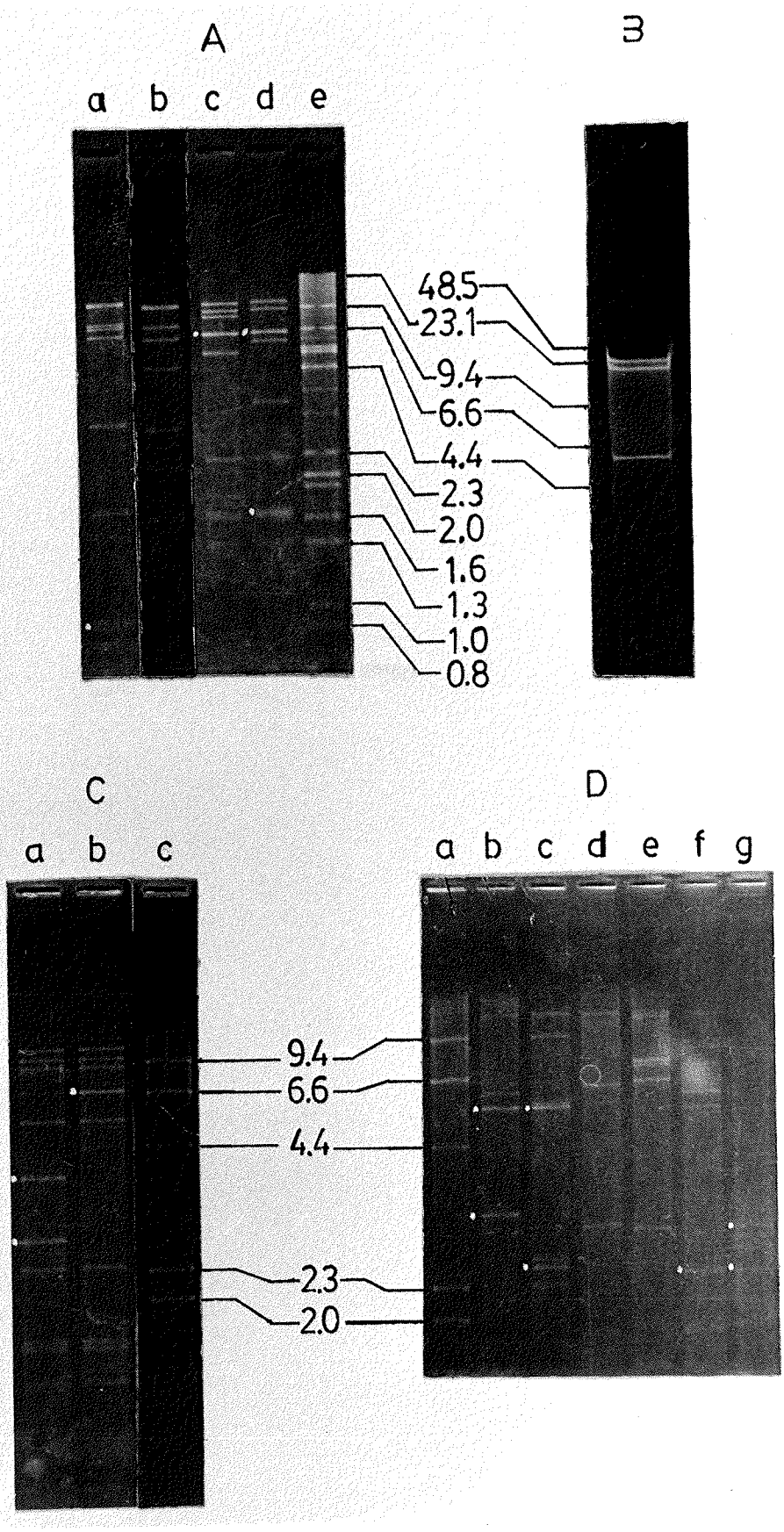

A
a b c d e f g
23.1
9.4
6.6
4.4
2.3
2.0
B
a b c
23.1
9.4
6.6
4.4
2.3
2.0
C
a b c d e f
D
a b c d e f
23.1
9.4
6.6
4.4
2.3
2.0

Figure 27. A) Digests produced by BglII (lane a), BglII/BalI (lane b), HincII (lane c), and HincII/BalI (lane d). Electrophoresis was for 4.5 h at 85 V through a 0.8% agarose gel. The size scale was based on HindIII and HindIII/EcoRI digests of lambda DNA (lane e). B) Digest produced by BalI. Electrophoresis was for 5 h at 60 V through a 0.8% agarose gel. The size scale was based on unrestricted lambda DNA and a HindIII digest of lambda DNA. C) Digests produced by HincII/SstII (lane a), and HincII (lane b). Electrophoresis was for 5 h at 90 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane c). D) Digests produced by BstEII (lane b), BstEII/ClaI (lane c), ClaI (lane d), BglII (lane e), BglII/SstI (lane f), and SstI (lane g). Electrophoresis was for 14 h at 40 V through a 0.8% agarose gel. The size scale was based on a HindIII digest of lambda DNA (lane a).


A
a b c d e
48.5
23.1
9.4
6.6
4.4
2.3
2.0
1.6
1.3
1.0
0.8
B
C
a b c
9.4
6.6
4.4
2.3
2.0
D
a b c d e f g

Blank leaf

DISCUSSION

DISCUSSION

The main objective of the research effort reported here was to derive a physical map for the mtDNA of the oomycete Achlya klebsiana. The mtDNA was found to be a 50.4 kb circular molecule of which between 19% and 25% is repeated in reverse order. The repeated regions are separated by regions of single copy sequence. This type of genome organization results in two isomeric forms of the molecule: head to tail and head to head orientation of the single copy regions with respect to each other. Both forms were found in about equal abundance in A. klebsiana, consistent with similar findings for A. ambisexualis and A. heterosexualis (Hudspeth et al, 1983; Shumard et al, 1986).

Among fungi in general, the Achlya mt genome is intermediate in size, falling between the smallest known genome size, the 17 kb mtDNA of a strain of Schizosaccharomyces pombe (Zimmer et al, 1984), and one of the largest known ones, the 115 kb mtDNA of Cochliobolus heterostrophus (Garber and Yoder, 1984). Among the Mastigomycotina (zoosporic fungi) only two other mtDNAs have been physically mapped. Allomyces macrogynus (Chytridiomycetes, Blastocladiales) contains a 56.1 kb mtDNA (Borkhardt and Delius, 1983), and Phytophthora infestans (Oomycetes, Peronosporales) contains a 36.2 kb mtDNA (Klimczak and Prell, 1984).

Among organelle DNAs, large inverted repeats are typical of the chloroplast DNAs of plants and green algae, but relatively rare in mtDNAs (Palmer, 1985). Besides Achlya, large inverted repeats have been found in the mt genomes of the yeast Kloeckera africana (Clark-Walker et al, 1981), the yeast Candida albicans (Wills et al, 1985), the protozoan Tetrahymena pyriformis (Goldbach et al, 1979), the protozoan Isospora gondii (Borst et al, 1984), and the plant Zea mays (Lonsdale et al, 1984). In the chloroplast DNAs, both large and small rRNA genes are completely encoded within the repeats, and in the mtDNAs of Achlya, Kloeckera, and Tetrahymena, rRNA genes are also associated with the repeats. In Achlya the repeats encode both small and large rRNA genes (Hudspeth et al, 1983; Boyd et al, 1984; Shumard et al, 1986). In K. africana the inverted duplications are 4.3 kb in size, are not separated by single copy sequences, and contain the large rRNA gene sequences only (Clark-Walker et al, 1981). Moreover, duplication of the large rRNA gene is incomplete such that the mt genome of K. africana contains only one full length copy of the ribosomal gene set. In the linear mtDNA of T. pyriformis the inverted repeats are terminally located and contain complete large rRNA genes only (Goldbach et al, 1979).

The advantage of having multiple copies of the genes encoding the RNA components of the protein-synthesizing machinery - rRNAs, 5S RNA, tRNAs - is well established and gene dosage is especially important during rapid growth (Long and Dawid, 1980). Thus, inclusion of rRNA genes in the inverted repeats of organelle DNAs may represent an adaptation for rapid growth. However, it has yet to be established that both copies of the rRNA genes in the organelle are functional.

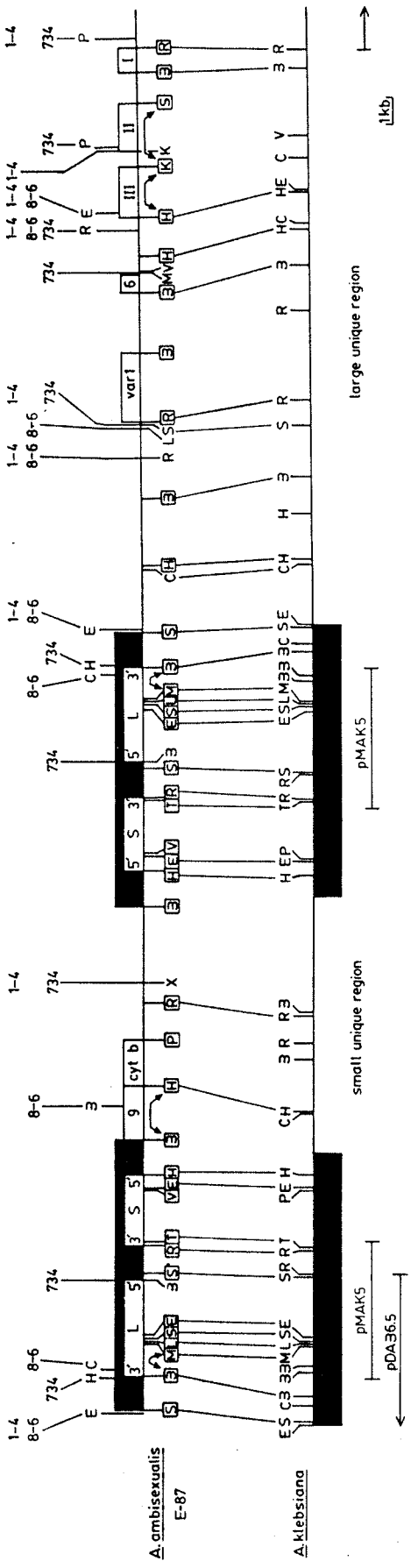
One property that inverted repeats are expected to confer is that of reversal of polarity of the single copy regions located between the repeats (Adelburg and Berquist, 1972; Guerineau et al, 1976; Kolodner and Tewari, 1979). This orientational or flip-flop isomerization is believed to occur through intramolecular recombination between aligned repeat regions. The polarity of the unique regions between the inverted repeats can be observed when restriction analysis shows asymmetric cleavage with respect to the inverted repeats (Gordon et al, 1981; Palmer, 1983). The presence of both orientations of unique regions in equimolar proportions was confirmed for A. klebsiana by appropriate restriction analysis. The same situation occurs in A. ambisexualis (Hudspeth et al, 1983). The presence of flip-flop isomers in about equimolar proportions in the population of Achlya mtDNA molecules indicates that homologous recombination is taking place

on a time scale comparable to that of the cell cycle. The fact that the axis about which flip-flop occurs is identical to the midpoint between any two homologous restriction sites within the repeats (Fig. 12, arrows), also argues for the occurrence of homologous recombination. Other mechanisms for generating flip-flop isomers would exactly reverse an entire unique region only by coincidence, whereas identity of midpoints is expected if homologous recombination is occurring (Hudspeth et al,1983). In addition, our mapping studies as well as studies with other Achlya species (Hudspeth et al, 1983; Shumard et al, 1986), and studies with chloroplast DNAs (Bedbrook and Kolodner, 1979), show that in all genomes with inverted repeats, the repeat arms are always identical. This is believed to be due to copy-choice correction between the two segments leading to homogenization (Kolodner and Tewari, 1979; Palmer, 1983). Indeed, intramolecular homologous recombination may be a universal feature of DNA molecules containing inverted repeats. Several other circular DNA molecules containing large inverted repeats have also been analysed for the presence of flip-flop isomers. These include the chloroplast genomes of common bean (Palmer, 1983), soybean (Palmer et al, 1984), the fern Osmunda (unpublished results of Palmer cited in Palmer, 1983), and the cyanelle DNA of Cyanophora paradoxa (Bohnert and Loffelhardt, 1982).

In each case it was found that the population of DNA molecules contains approximately equimolar amounts of the two flip-flop isomers. Similarly, the 2 um plasmid of Saccharomyces cerevisiae, which contains an inverted repeat of 599 bp, exists as two flip-flop isomers (Guerineau et al, 1976). In addition, the linear genomes of several Herpes simplex viruses contain two sets of inverted repeats, and all four possible flip-flop isomers are present in equimolar proportions (Roizman, 1979). In the case of the S. cerevisiae 2 um plasmid, it has been found that recombination between the repeat regions is mediated by a specialized site-specific recombination protein encoded by a gene (FLP) carried on the plasmid itself (Broach, 1982). The functional importance of flip-flop isomerization, if any, has not yet been determined. One suggested role of the switching system in the S. cerevisiae 2 um plasmid is that interconversion may act as a genetic switch allowing alternate transcription of flanking genes (Broach, 1982). It is possible that in mitochondria, flip-flop isomerization is just a physical consequence of the presence of inverted repeats in the mtDNA and has no functional significance.

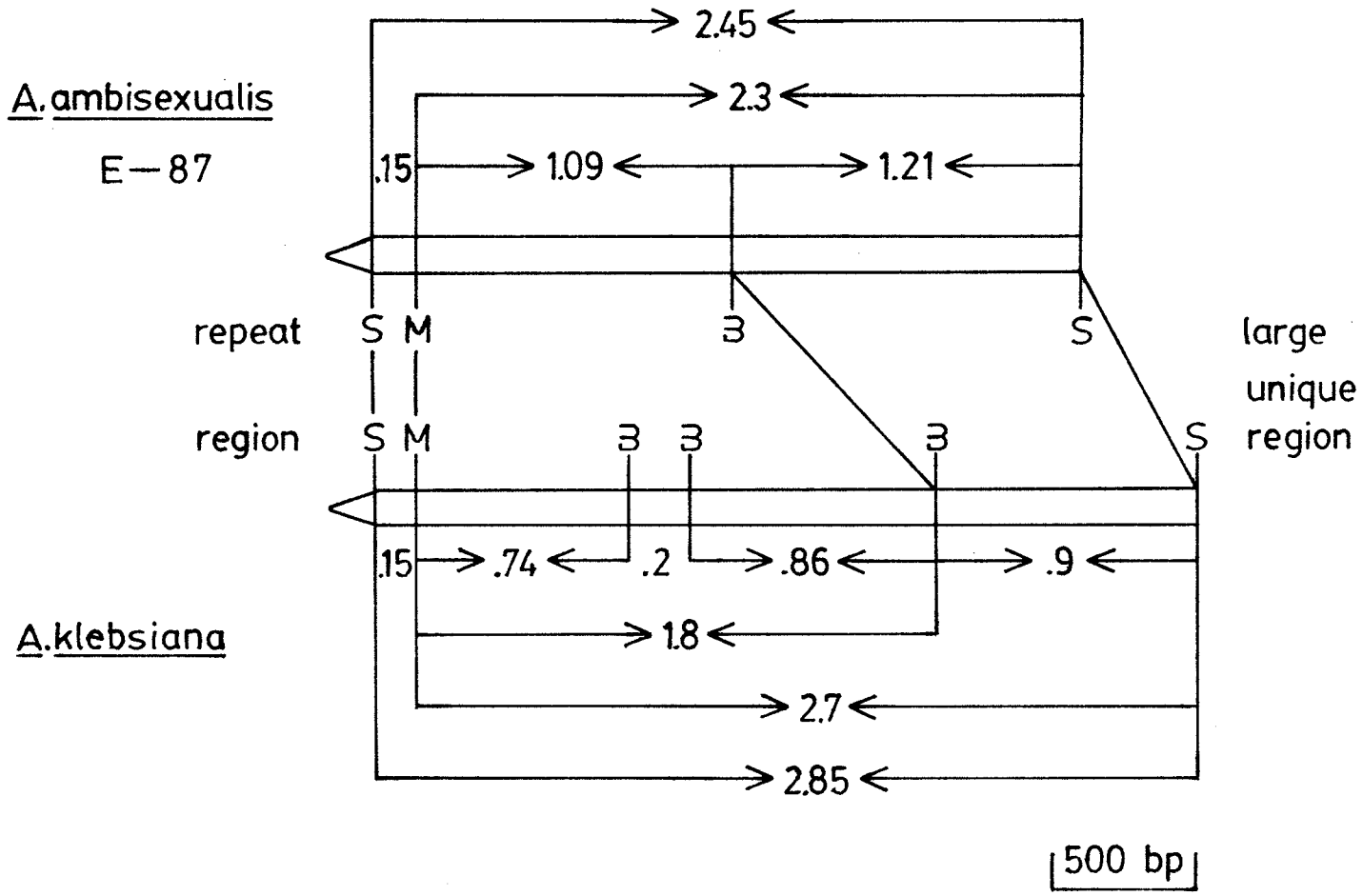
The physical maps of the mt genomes of four heterothallic Achlya strains have been analysed and compared (Shumard et al, 1986). The strains include A. ambisexualis E-87, A. ambisexualis 734, A. heterosexualis 1-4, and A. heterosexualis 8-6 (hereafter referred to as E-87, 734, 1-4, and 8-6). A minimal composite map was derived for those strains by retaining only the 41 restriction sites held in common by all four strains and by selecting the minimum strain distance between any two common sites (as determined by systematic side-by-side comparisons of DNA fragment lengths generated by common restriction sites). Analysis of the DNA fragment length differences revealed four regions of the mt genome subject to insertion events as compared to the minimal composite map. Of these four regions, two occur in the large unique region, one in the small unique region, and one region is in the repeats (Fig. 28, sites joined by arrowheads). Comparison of the A. klebsiana physical map to those of the heterothallic strains (Fig. 28) indicates that insertion/deletion events appear to have occurred in the same regions of the A. klebsiana mtDNA as in the other Achlya strains. This would account, in part, for some of the common sites between the strains being out of register. Side-by-side length comparisons of common DNA fragments would be required to determine more precise location and actual size of any insertion/

Figure 28. Alignment of the A. klebsiana and A. ambisexualis E-87 restriction maps. Maps are linearized at one repeat/large unique region junction. Solid areas ■ indicate minimum extent of inverted repeat regions. Sites common to both A. klebsiana and E-87 are joined by vertical or near vertical lines. Boxed sites on the E-87 map indicate sites common to E-87, A. ambisexualis 734, A. heterosexualis 1-4, and A. heterosexualis 8-6. Sites unique to one of, or common to two or three of E-87, 734, 8-6, and 1-4, are indicated above the E-87 map. Sites on the E-87 map joined by arrowheads indicate regions of insertion/deletion events. Regions that hybridized to specific gene probes are indicated on both maps: I, II, III, are cytochrome c oxidase subunits I, II, and III; 6 and 9 are ATPase subunits 6 and 9; cyt b is apocytochrome b; var1 is mito-ribosomal protein var1; S and L are small and large rRNAs; 5' and 3' indicate the respective termini of the rRNA genes. Cloned fragments of A. klebsiana mtDNA are indicated by pMAK5 (BclI4) and pDAB6.5 (EcoRI5). Restriction sites: B, BglII; C, ClaI; E, BstEII; H, HpaI; K, KpnI; L, SalI; M, BamHI; P, PstI; R, EcoRI; S, SstI; T, SstII; V, PvuII; X, XhoI. Restriction and gene mapping for E-87, 734, 8-6, and 1-4, from Hudspeth et al, 1983, and Shumard et al, 1986.


A. ambisexualis
E-87
A. klebsiana
large unique region
small unique region
pMAK5
pDA36.5
1kb

deletion event between the A. klebsiana and the heterothallic strains' genomes. In one case, however, that of the insertion in the repeat region of 734, 1-4, and 8-6 relative to E-87, comparison of the E-87 and A. klebsiana maps reveals the presence of a relatively large insertion in the A. klebsiana mtDNA. This region is defined by the common BamHI site and the common BglII site (proximal to the large unique region) in the repeats of the two genomes (between 29.0-32.5 m.u. and 79.6-83.2 m.u. in the A. klebsiana map shown in Fig. 12). This region is internal to fragment SstI5 of E-87 and fragment SstI4 of A. klebsiana and a detailed size comparison of this region is shown in Fig 29. The BamHI/BglII distance is 1.09 kb in E-87 but 1.8 kb in A. klebsiana. Thus the data would indicate a 0.71 kb insertion in this region of the A. klebsiana mtDNA relative to E-87. This would be in the range of insertion sizes (0.62-0.86 kb) detected in the analogous regions of the 734, 1-4 and 8-6 mtDNAs. However, a 0.71 kb insertion would account for too much of the DNA fragment length variation in this region since the absolute difference between SstI5 of E-87 and SstI4 of A. klebsiana is only 0.40 kb. It can be seen from Fig 29 that the additional 0.31 kb is due to a difference in the BglII-SstI distance between the two maps (1.21 kb in E-87, but only 0.9 kb in A. klebsiana). As no insertion/deletion events were detected in this

Figure 29. Length comparison between A. ambisexualis E-87 mtDNA fragment SstI5 and A. klebsiana mtDNA fragment SstI4. Distances (kb) between restriction sites are shown. Restriction sites: B, BglII; M, BamHI; S, SstI. Restriction data for E-87 fragment SstI5 from Hudspeth et al, 1983.


A. ambisexualis
E—87
2.45
2.3
.15
1.09
1.21
repeat
region
S
M
B
S
large
unique
region
S
M
B
B
B
S
.15
.74
.2
.86
.9
A. klebsiana
1.8
2.7
2.85
500 bp

region in any of the heterothallic strains, it is likely that this 0.31 kb difference is due to mapping inaccuracies between E-87 and A. klebsiana. In addition, there are two other BglII sites in this region of the A.klebsiana mt genome, either of which could be the common site shared with E-87. However one would then have to postulate an insertion/deletion event between this common BglII site and the common SstI site (proximal to the large unique region) and as noted above, none has been shown to occur in any of the heterothallic strains. Thus the most likely interpretation is that an insertion of approximately 0.4-0.7 kb has occurred in the repeat region of the A. klebsiana mt genome (29.0-32.5 m.u. and 79.6-83.2 m.u., Fig. 12) relative to the analogous region in the mt genome of E-87, similar to the insertions in mtDNAs of the heterothallic strains 734, 1-4, and 8-6.

The colinearity of the Achlya mtDNAs (Fig. 28) allows the study of divergence among their genomes by using equations that relate the number of shared restriction sites to sequence divergence (Brown et al, 1979; Nei and Li, 1979). Only restriction enzymes whose sites were mapped in all five Achlya strains are used in the analysis. All restriction enzymes, except BstEII, recognize specific hexanucleotide sequences and thus the analysis is limited to the gain or loss of sites whose recognition sequences require six nucleotides.

The BstEII sites were retained in the analyses because the additional one nucleotide in its heptanucleotide recognition sequence (5'-GGTNACC-3'; N can be any one of G, A, T, or C) is completely degenerate. Analysis of the four heterothallic strains, whether based on pairwise comparisons or comparison of total site variations involving all four strains, clearly indicated the relative conservation of the repeat sequences (Shumard et al, 1986). The results indicated an overall sequence divergence of 6-7%. A detailed analysis of regions within the genome, however, revealed that divergence within the repeat was about one-third of that in single copy sequence and further that divergence within rRNA genes was one-fifth of that in non-ribosomal repeat sequences. This indicates that repeat sequence conservation may be due to the encoding of rRNA genes. To see whether this pattern was retained in a more distant relationship, similar calculations were performed in a comparison between A. klebsiana, a homothallic species, and E-87, which is heterothallic. The alignment shown in Fig 28 was used for the calculations (sites shared by A. klebsiana and E-87 are joined by vertical or near vertical lines). Since the two repeat arms are identical most of the divergence values are derived from the total genome less one inverted repeat arm (Table 7). As seen in Table 7, the A. klebsiana and E-87 mtDNAs differ by an average

of 12.5% in overall nucleotide sequence divergence. When only one arm of the inverted repeat is included the divergence increases to 13.6%. Comparison of genomic regions reveals that the inverted repeats are more conservative than single copy sequences. Repeat regions have diverged by 10.0% while single copy sequences have done so by 16.3%, a 1.6 fold difference (Table 7). Further, there is a 2.5 fold difference between repeat rDNA (7.4% divergence) and repeat non-rDNA (18.3% divergence) sequences (Table 7). Thus the data appear to support the conclusion that the repeat sequence conservation in Achlya is due to the encoding of rRNA genes and that this phenomenon applies over fairly large evolutionary distances. Exclusion of the rDNA from the calculations significantly increases the sequence divergence values between the remaining 85% (in sequence complexity) of the genome. Without rRNA genes, overall divergence rises from 13.6% to 16.6%. However, the paucity of restriction sites in the repeat non-rDNA region limits the strength of the conclusion concerning the involvement of rRNA genes in repeat conservation. Nevertheless, base substitutions in Achlya are non-random, the inverted repeat evolving more slowly than the single copy DNA. Similar conclusions were reached in a study of legume chloroplast genomes (Palmer et al, 1983).

Table 7. Analysis of sequence divergence between *A. klebsiana* and *A. ambisexualis* E-87.

Genomic region compared	Restriction sites compared	Restriction sites shared	Percent sequence divergence
Total genome	72	34	12.5
Total genome less one repeat	52	23	13.6
Total repeat - one copy	20	11	10.0
repeat rDNA - one copy	14	9	7.4
repeat non-rDNA - one copy	6	2	18.3
Total single copy	32	12	16.3
small single copy	8	2	23.1
large single copy	24	10	14.6
Non-rDNA less one repeat	38	14	16.6
Non-rDNA total genome	44	16	16.9

Restriction sites shared/restriction sites compared = S;

Number of nucleotides recognized = N;

Percent sequence divergence = $-100(\ln S)/N$ (W. Brown et al, 1979; Nei and Li, 1979).

The colinearity of the A. klebsiana and E-87 mt genomes means that the preliminary gene map established for E-87 (Shumard et al, 1986), including the genes for ATPase 6, ATPase 9, cyt b, COI, COII, COIII, var1, and the small and large rRNAs, is probably true for A. klebsiana as well (Fig. 28). Construction of detailed physical maps thus simplifies genetic mapping in closely related species and makes possible fruitful evolutionary comparisons.

Two restriction fragments from the A. klebsiana mt genome (BclI4 and EcoRI5) were cloned into plasmid vectors. Plasmid pMAK5 contains fragment BclI4 which mapped totally within the repeat regions (see Figs. 21 and 28). Extensive restriction mapping of pMAK5 showed no differences between the cloned fragment and the A. klebsiana map in this region. As can been seen in Fig 28, pMAK5 contains most of the sequence that hybridized to the large rRNA gene probe in A. ambisexualis E-87. Plasmid pDAB6.5 contains fragment EcoRI5 (see Figs. 21 and 28). Restriction mapping of pDAB6.5 showed no differences between the cloned fragment and the A. klebsiana map in this region, except in digestions involving BclI and ClaI. In the EcoRI/BclI and EcoRI/ClaI codigestions of pDAB6.5 DNA (Fig. 19, lanes f and h), it can be seen that BclI and ClaI do not cleave as predicted (see Fig. 21).

It is likely that this was due to methylation of their recognition sites by the *dam* methylase of *E. coli* JM103, which methylates the internal adenine in the sequence 5'-GATC-3' (Wilson and Young, 1975; Pirotta, 1976). *BclI* recognizes the sequence 5'-TGATCA-3' (Bingham et al, 1978), and *ClaI* recognizes the sequence 5'-ATCGAT-3' (Mayer et al, 1981). The *ClaI* site in the *EcoRI5* fragment of *A. klebsiana* mtDNA must therefore have a G on its left or C on its right such that the *dam* methylase recognition site would be complete. *BclI* and *ClaI* are inhibited when the internal adenine of their recognition sites is methylated (Dreiseikelmann et al, 1979; McClelland, 1981). As can be seen in Fig 28, *pDAB6.5* contains the full sequence that hybridized to the large rRNA gene probe in *A. ambisexualis* E-87, as well as the region which contains the strain-specific repeat insertion. It has not been determined whether this insertion interrupts the large rRNA gene sequence or is merely located at its 3' terminus in those *Achlya* strains containing it (Shumard et al, 1986). R-loop analysis and/or DNA sequence analysis would be required to determine if the strain-specific repeat insertion in *Achlya* is analogous to optional large rRNA introns described in other fungi (see Historical section 3.5). The availability of clones containing this region will facilitate this analysis.

As discussed above (Results section 3.2), pDAB6.5 also contains the region in which the junction between the repeat regions and the large unique region lies (see Figs. 21 and 28). This region might be of interest with respect to the mechanism of flip-flop isomerization since it may be here that intramolecular recombination begins.

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